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<b>(54) Title:</b> CHIMERIC FUCOSYLTRANSFERASE POLYPEPTIDES AND NUCLEIC ACID SEQUENCES ENCODING THEM <b>(57) Abstract</b> <p>A method for isolating a gene, comprising: (i) isolating a cell possessing a post-translational characteristic of interest, said post-translational characteristic being the presence of a membrane-bound oligosaccharide or polysaccharide of interest on the surface of said cell, the presence of a soluble oligosaccharide or polysaccharide of interest in an extract of said cell, or the presence of a particularly glycosyltransferase activity in an extract of said cell; (ii) creating a genetic library of either cDNA or genomic DNA from the genetic material of said isolated cell; (iii) transforming host cells with said genetic library; and (iv) screening said transformed host cells for a host cell containing said post-translational characteristic, thereby obtaining a cell containing said gene, is disclosed. The method can be used to obtain genes encoding glycosyltransferases.</p>		

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TITLE OF THE INVENTION

"CHIMERIC FUCOSYLTRANSFERASE POLYPEPTIDES AND NUCLEIC ACID SEQUENCES ENCODING THEM".

BACKGROUND OF THE INVENTION

This work was supported by National Institutes of Health Grants GM47455 and K11 DK02189, and the U.S. Government may have rights in this application.

Field of the Invention:

The present invention relates to methods and products for the synthesis of oligosaccharide or polysaccharide structures, on glycoproteins, glycolipids, or as free molecules. The present invention also relates to novel chimeric fucosyltransferases.

Discussion of the Background:

Carbohydrates are an important class of biological compounds which are remarkable for their structural diversity. This diversity is not random but rather consists of specific sets of oligosaccharide structures that exhibit precise tissue-specific and developmental expression patterns. In cells carbohydrates function as structural components where they regulate viscosity, store energy, or are key components of cell surfaces. Numerous site specific intercellular

interactions involve cell surface carbohydrates. For example, union of sperm and egg as well as the implantation of fertilized egg are both mediated by cell surface carbohydrates. Likewise, a number of proteins that function as cell adhesion molecules, including GMP-140, Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1), and lymphocyte adhesion molecules like Mel-14, exhibit structural features that mimic lectins, and are now known to bind specific cell surface carbohydrate structures (Feizi, Trends Biochem. Sci. (1991) 16:84-86). Glycosylated proteins as tumor-associated antigens are now being used to identify the presence of numerous carcinomas. Even isolated oligosaccharides have been found to exhibit biological activity on their own.

Specific galactose oligosaccharides are known to inhibit the agglutination of uropathogenic caliform bacteria with red blood cells (U.S. Patent No. 4,521,592). Other oligosaccharides have been shown to possess potent antithrombic activity by increasing the levels of plasminogen activator (U.S. Patent No. 4,801,583). This same biological activity has been used, by binding oligosaccharides, in conjunction with an amino glycoprotein, in medical instruments to provide medical surfaces which have anticoagulation effects (U.S. Patent No. 4,810,784). Still other oligosaccharides have found utility as gram positive antibiotics and disinfectants (U.S. Patent Nos. 4,851,338 and 4,665,060). Further, oligosaccharides have been used as bacteria receptor



sites in the diagnosis and identification of specific bacteria (U.S. Patent Nos. 4,657,849 and 4,762,824).

It is also well recognized that oligosaccharides have an influence on the protein or lipid to which they are conjugated (Rademacher et al, Ann. Rev. Biochem., (1988) 57:785).

Specific oligosaccharides have been shown to influence proteins' stability, rate of in vivo clearance from blood stream, rate of proteolysis, thermal stability and solubility. Changes in the oligosaccharide portion of cell surface carbohydrates have been noted in cells which have become cancerous. Other oligosaccharide changes have been detected during cell differentiation (Toone et al, Tetrahedron Report (1989) 45(17):5365-5422). As such, the significance of oligosaccharides to biological function cannot be understated.

The fundamental role of these materials in molecular biology has made them the object of considerable research, in particular, considerable efforts have been made in organic synthesis to synthesize these materials. Although synthetic approaches to making carbohydrates are quite developed, this technique suffers notable difficulties which relate to the selective protection and deprotection steps required in the available synthetic pathways. These difficulties, combined with difficulties associated with isolating and purifying carbohydrates, and determining their structures, has made it essentially impossible for synthetic organic chemistry to economically produce valuable carbohydrates.

Enzyme-mediated catalytic synthesis would offer dramatic advantages over the classical synthetic organic pathways, producing very high yields of carbohydrates (e.g., oligosaccharides and/or polysaccharides) economically, under mild conditions in aqueous solution, and without generating notable amounts of undesired side products. Such enzymes, which include glycosyltransferases, are however difficult to isolate, especially from eukaryotic, e.g., mammalian sources, because these proteins are only found in low concentrations, and are membrane-bound.

As of 1987, standard molecular cloning approaches which require amino acid sequence information or anti-glycosyltransferase antibodies, had been successfully used to isolate just two eukaryotic, e.g., mammalian glycosyltransferase cDNAs, corresponding to  $\beta(1,4)$ galactosyltransferase (in 1986) and  $\alpha(2,6)$ sialyltransferase (in 1987). In light of the above-noted considerable value of carbohydrates, there is accordingly a strongly felt need for an improved method for isolation of additional glycosyltransferase genes and cDNAs and for their use in carbohydrate synthesis.

SUMMARY OF THE INVENTION

Accordingly, it is an object of this invention to provide a method for readily isolating eukaryotic, e.g., mammalian glycosyltransferase genes and cDNAs.

It is another object of this invention to provide a method to modify these isolated genes and cDNAs to obtain correspondingly modified glycosyltransferases.

It is another object of this invention to provide these unmodified and modified isolated genes and cDNAs, and to use them, for example, in modifying cell surface oligosaccharide structure via gene transfer approaches or via in vitro glycosylation reactions.

The inventor has now discovered a gene transfer approach which satisfies all of the above-noted objects of this invention, and other objects which will be seen from the description of the invention given hereinbelow. The present methodology takes advantage of existing information about substrate and acceptor properties of glycosyltransferases and makes use of the numerous antibody and lectin reagents that are specific to the cell surface-expressed oligosaccharide products of these enzymes. The inventors have also discovered that useful chimeric fucosyltransferases can be prepared from enzymes so obtained.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1, 2, 3, 4, 5, 6, and 7 provide six DNA sequences provided by the invention, encoding glycosyltransferases.

Figure 1 shows the DNA sequence encoding a protein capable of functioning as a GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)-D-GlcNac(/Glc)- $\alpha$ -(1,3/1,4)-fucosyltransferase (Lewis fucosyltransferase, Fuc-TIII) (SEQ ID NO:1) and the amino acid sequence of the encoded protein (Fuc-TIII) (SEQ ID NO:2).

Figure 2 shows the DNA sequence encoding a mouse UDP-Gal: $\beta$ -D-Gal-(1,4)-D-GlcNac  $\alpha$ (1,3)-galactosyltransferase (SEQ ID NO:3) and the encoded protein (SEQ ID NO:4). Figure 3 shows the DNA sequence encoding a human GDP-Fuc: $\beta$ -D-galactoside  $\alpha$ (1,2)-fucosyltransferase (SEQ ID NO:5) and the amino acid sequence of the encoded protein (SEQ ID NO:6).

Figures 4 and 5 provide DNA sequences (SEQ ID NO:7 and SEQ ID NO:9, respectively) encoding a GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNac $\alpha$ (1,3)-fucosyltransferase (Fuc-TIV) and the encoded protein (Fuc-TIV) (SEQ ID NO:8). Figure 5 also shows the amino acid sequence of the Lewis fucosyltransferase (Fuc-TIII) (SEQ ID NO:2).

Figure 6 provides a DNA sequence (SEQ ID NO:10) encoding a GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNac $\alpha$ (1,3)-fucosyltransferase (Fuc-TV) (numbered upper nucleotide strand) and its corresponding protein sequence (Fuc TV) (SEQ ID NO:11), together with the DNA sequence of the Lewis blood group fucosyltransferase (Fuc-TIII) (unnumbered lower sequence).

Amino acid differences with the Lewis fucosyltransferase are indicated by inclusion of Lewis amino acids below the Lewis DNA sequence (SEQ ID NO:12). The transmembrane domain of the fucosyltransferase is underlined.

Figure 7 provides a DNA sequence of the coding portion of the genomic DNA insert in pCDNA1- $\alpha$ (1,3)Fuc-TVI (SEQ ID NO:13), and parts of the 5' and 3' regions of that gene. DNA sequence comparison between the GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNac $\alpha$ (1,3)-fucosyltransferase (Fuc-TVI) encoded by the genomic DNA fragment in pCDNA1- $\alpha$ (1,3)Fuc-TVI (labeled  $\alpha$ (1,3)FT DNA) and the Lewis blood group fucosyltransferase (Fuc-TIII) (labeled Lewis DNA) (SEQ ID NO:12) is also shown in Fig. 7. Positions of DNA sequence identity are denoted by a vertical line (|) between identical nucleotides at similar positions. Positions where the sequences are out of register are denoted by (.). The derived protein sequence of Fuc-TVI, in single letter code, and labeled  $\alpha$ (1,3)FT AA, is indicated above its DNA sequence (SEQ ID NO:14).

Figure 8 presents flow cytometry profile histograms of COS-1 cells transfected with different  $\alpha$ (1,3)fucosyltransferase gene expression vectors, or with control vectors, and subjected to analysis with monoclonal antibodies directed against cell surface oligosaccharide determinants. COS-1 cells were transfected either with plasmid pCDM7 (pCDM7), with plasmid pCDNAI(pCDNAI), with pCDM7 containing DNA sequences encoding the

$\alpha(1,3/1,4)$ fucosyltransferase depicted in Figure 1 (the Lewis fucosyltransferase, also known as Fuc-TIII, SEQ ID NO:2), or with pCDNAI containing DNA sequences encoding the  $\alpha(1,3)$ fucosyltransferase depicted in Figure 4 (Fuc-TIV, SEQ ID NO:8), the  $\alpha(1,3)$ fucosyltransferase depicted in Figure 6 (Fuc-TV, SEQ ID NO: 11), or the  $\alpha(1,3)$ fucosyltransferase depicted in Figure 7 (Fuc-TV<sup>TVI</sup>, SEQ ID NO:14). Three days after transfection, the cells were harvested, stained with monoclonal antibodies (shown at the top left within the figure) directed against the H (anti-H), Lewis x (anti-Lex), sialyl Lewis x (anti-sLex), Lewis a (anti-Lea), or sialyl Lewis a (sLea) oligosaccharide determinants, and then stained with a fluorescein-conjugated second antibody. The cells were then subjected to analysis by flow cytometry. The histograms represent the mean fluorescent intensities of the antigen-positive cells in each transfectant population (approximately 25% to 30% of the cells are transfected and express the positive cell surface markers). Methods for these analyses have been described in detail in Lowe et al, J. Biol. Chem., (1991), 266:17467-17477, Weston et al, J. Biol. Chem., (1992), 267:4152-4160, Lowe et al, Cell, (1990), 63:475-484, and Ernst et al, J. Biol. Chem., (1989), 264:3436-3447.

Figures 9a and b show a comparison of protein and DNA sequences of Fuc-TIII and Fuc-TV<sup>TVI</sup>. Fig. 9a, Fuc-TIII and Fuc-TV<sup>TVI</sup> are schematically represented by open rectangles. The transmembrane domain of each enzyme is denoted by a striped




box (  ). Vertical lines within the rectangles denote the positions of amino acid residues that differ between the two enzymes. These differences are concentrated in the "hypervariable" segment between the *Afl* III and *Bgl* II restriction sites shown under the rectangles. The carboxyl-terminal regions of these enzymes, distal to the *Bgl* II site, differ at 7 positions. The relative positions of these differences, and their nature (six non-identical amino acids and one amino acid/insertion; identified in single letter amino acid code) are also indicated in the figure. Sites of potential asparagine-linked glycosylation are indicated by  . The disaccharide portions of the potential oligosaccharide acceptors are also shown in the figure. "LacNAc" denotes the type II precursor *N*-acetylactosamine. LNB-I denotes the type I precursor lacto-*N*-biose I. Arrows near the disaccharide moieties point to the hydroxyl group that will be modified by fucosylation. R denotes the underlying glycoconjugate that displays the disaccharide. R' represents either a hydroxyl group in the neutral precursors for Lewis x or Lewis a products, or  $\alpha(2,3)$ -linked sialic acid in the sialylated precursor for the sialyl Lewis x or sialyl Lewis a products. Fuc-TIII (top) can utilize GDP-fucose to form products from both type II and type I precursors, whereas Fuc-TVI (bottom; shaded to match Figs. 10, 11a and b, and 12a and b) can utilize GDP-fucose to form products only from the type II precursors.

Fig. 9b, Subdomains within the "hypervariable" segments of Fuc-TIII and Fuc-TVI. The DNA and derived protein sequences of the two enzymes are aligned. The upper DNA and protein sequences correspond to Fuc-TIII. The lower DNA and protein sequences correspond to Fuc-TVI. Dotted lines (....) indicate positions in one enzyme that do not have a counterpart in the other. Residues are numbered beginning with the initiator methionine codon of Fuc-TIII (residue number 1). Non-identical amino acid residues are *shaded*. Underlined DNA sequences correspond to the synthetic oligonucleotides used to create or destroy restriction sites (positions and identity indicated above, for Fuc-TIII, or below, for Fuc-TVI) that define the boundaries of each subdomain. The nature of the DNA sequence changes needed to create these sites are denoted above (Fuc-TIII) or below (Fuc-TVI) the wild type DNA sequence. As noted in the text, these changes maintain the wild type protein sequence of each enzyme.

Figure 10 shows flow cytometry histograms of COS-7 cells transfected with recombinant Fuc-T chimeras generated via carboxyl-terminal domain exchanges. Fuc-TIII (*open* rectangles) and Fuc-TVI (*shaded* rectangles) are illustrated as in Figure 9a. Recombinant chimeras derived by restriction fragment exchange procedures (see Experimental Procedures) are composed of segments of Fuc-TVI (*shaded* areas) and Fuc-TIII (*open* areas). Transmembrane segments are denoted by the



darkly shaded regions near the NH<sub>2</sub>-termini of each schematically represented enzyme. A symbol (  ) denotes the positions of potential asparagine-linked glycosylation sites. Relative positions of the five subdomains in the "hypervariable" region are numbered. Positions of the *Bgl*III and *Bst*XI sites used to create the chimeras in this figure are indicated below each schematically represented enzyme. COS-7 cells transfected with vectors encoding each enzyme, or a negative control vector (pcDNAI) were stained with the monoclonal antibodies indicated in the legend in the figure, and analyzed by flow cytometry. Histograms display the fraction of antigen-positive cells (normalized for transfection efficiencies, as described in the Examples, and represent the average of either two or three experiments. The mean fluorescence intensity (M.F.I.) of the antigen-positive cells are indicated by the numbers at the left of each histogram, above the arrow (↑) at the bottom of each panel. Numbers in parenthesis in this column denote the mean fluorescence intensity of the antigen-negative cells whenever the experiment yielded no antigen-positive cells.

Figures 11a and b show flow cytometry histograms of COS-7 cells transfected with recombinant Fuc-T chimeras generated via single subdomain exchanges. Symbols, legends, and nomenclature are identical to that used in Figure 10. The five subdomains in the "hypervariable" region of each enzyme are numbered. COS-7 cells transfected with vectors encoding

each enzyme, or a negative control vector (pcDNAI) were stained with the monoclonal antibodies indicated in the legend in each panel, and were analyzed by flow cytometry. Histograms display the fraction of antigen-positive transfected COS-7 cells (normalized for transfection efficiencies, as described in Experimental Procedures), and represent the average of either two or three experiments. Mean fluorescence intensities (M.F.I.) of antigen-positive cells are indicated by the numbers at the left of each histogram, above the arrow (↑) at the bottom of each panel. Numbers in parenthesis in this column denote the mean fluorescence intensity of the antigen-negative cells whenever the experiment yielded no antigen-positive cells. Figure 11a, single subdomains from Fuc-TVI (*shaded areas*) installed into Fuc-TIII (*open rectangles*). Figure 11b, single subdomains from Fuc-TIII (*open rectangles*) installed into Fuc-TIII (*shaded rectangles*).

Figures 12a and b show flow cytometry histograms of COS-7 cells transfected with recombinant Fuc-T chimeras generated via multiple subdomain exchanges. Symbols, legends, and nomenclature are identical to that used in Figures 10 and 11a and b. The five subdomains in the "hypervariable" region of each enzyme are numbered. COS-7 cells transfected with vectors encoding each enzyme, or a negative control vector (pcDNAI) were stained with the monoclonal antibodies indicated in the legend in each panel, and were analyzed by flow

cytometry. Histograms display the fraction of antigen-positive transfected COS-7 cells (normalized for transfection efficiencies, as described in the Examples), and represent the average of either two or three experiments. Mean fluorescence intensities (M.F.I.) of antigen-positive cells are indicated by the numbers at the left of each histogram, above the arrow (↑) at the bottom of each panel. Numbers in parenthesis in this column denote the mean fluorescence intensity of the antigen-negative cells whenever the experiment yielded no antigen-positive cells. Figure 12a, top set of histograms, multiple subdomains from Fuc-TVI (*shaded areas*) installed into Fuc-TIII (*open rectangles*) (chimeras Fuc-TC15, Fuc-TC16, and Fuc-TC19). Figure 12a, bottom histogram, subdomains 4 and 5 from Fuc-TV (*lightly shaded areas*) replace subdomains 4 and 5 in Fuc-TIII (chimera Fuc-TC21). Figure 12b, single subdomains from Fuc-TIII (*open rectangles*) installed into Fuc-TIII (*shaded rectangles*) (chimeras Fuc-TC17, Fuc-TC18, and Fuc-TC20).

Figure 13 shows protein sequence alignments of Fuc-TIII, Fuc-TV and Fuc-TVI across subdomains 4 and 5. The protein sequences of subdomains 4 and 5 of Fuc-TIII, Fuc-TV and Fuc-TVI are shown in the single letter code. Numbers at the left indicate the residue number of the leftmost amino acid of each peptide sequence. *Dashed lines* represent positions in Fuc-TV and FucTVI that are identical to corresponding residues

in Fuc-TIII. Non-identical residues are explicitly indicated by the single letter amino acid designation. Residues present in Fuc-TVI, but not found in either Fuc-TV or in Fuc-TIII ("Unique" to Fuc-TVI) are displayed below, amongst residues (indicated by \*) that are common to at least two of the three enzymes.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Generally, the present invention provides a method for isolating a gene and/or a cDNA from a cell, by using a post-translational characteristic of the cell. The cell from which this gene and/or cDNA may be isolated may be either a cell from a unicellular or a multicellular organism.

In the context of the present invention, a post-translational characteristic of a certain cell is defined by the ability of that cell to modify a protein or a lipid by an enzymatic process that covalently attaches to this protein or lipid one or more monosaccharides, or an enzymatic process that specifically removes such substituents from a protein or lipid molecule.

In one embodiment, the method comprises the following four basic steps:

- (i) identifying for use as a genetic donor, a eukaryotic (e.g. mammalian) cell possessing a post-translational characteristic of interest i.e. a particular membrane-bound oligosaccharide or polysaccharide (i.e. a glycoprotein or

glycolipid), soluble oligosaccharide or polysaccharide, or a particular enzymatic activity (vide infra);

(ii) creating a genetic library of either cDNA or genomic DNA from the genetic material of the donor eukaryotic (e.g. mammalian) cell;

(iii) identifying a specific eukaryotic host suitable as a recipient for gene transfer, and transforming this eukaryotic, (e.g. mammalian), host cells with this genetic library; and

(iv) screening the transformed host cells for host cells possessing the post-translational characteristic of interest.

The host cell which now possesses this post-translational characteristic contains genetic information related to the post-translational characteristic of interest. Using the techniques set forth below this genetic information (gene) can then be retrieved from the transformed host cell and used by standard approaches i.e., Axel et al (U.S. Patent No. 4,634,665) or Gilbert et al (U.S. Patent No. 4,411,994) to produce large quantities of the gene product, i.e., the glycosyltransferase, responsible for the post-translational characteristic.

In step (i) above the donor eukaryotic (e.g. mammalian) cell is chosen on the basis of detecting a specific enzymatic activity in an extract of the cell or detecting a membrane-bound or soluble oligosaccharide or polysaccharide of the cell.

Thus in one embodiment the enzymatic activity which is detected in the cell extract can be enzymatic activity attributable to an animal enzyme which post-translationally modifies proteins, lipids, or oligosaccharides by glycosylation or glycosyl modification. This enzymatic activity may be detected by using a substrate specific for one of these enzymes. Such substrates are known.

In another embodiment, in step (i) above a cell is chosen on the basis of detection of a specific cellular membrane-bound oligosaccharide and/or polysaccharide.

In another embodiment, the cell in step (i) is chosen on the basis of detecting the presence of a soluble oligosaccharide or polysaccharide in an extract of the cell, or released by the cell in soluble form.

The present invention provides a novel gene transfer approach designated to isolate genes from an organism without requiring that amino acid sequence information be obtained about the gene product or that an antibody specific to the gene product be available. For example, if a gene encoding a particular enzyme is sought, a series of cultured cell lines or tissues are screened by known and standard methods to identify one or more cell lines or tissues containing an expressible gene of interest by detecting in an extract of the cell or tissue specific enzymatic activity (corresponding to the enzyme of interest and thus the cell contains and/or expresses a gene for the enzyme sought). If an

oligosaccharide or polysaccharide membrane component of the cell is of interest, a cell line or tissue possessing such a membrane characteristic is isolated. If a soluble oligosaccharide or polysaccharide is of interest, a cell line or tissue possessing a soluble oligosaccharide or polysaccharide detectable in an extract thereof is isolated.

Once such a cell line or tissue has been identified, a genetic library based on this isolated cell is created. This genetic library may be either cDNA or genomic DNA. In a preferred embodiment, if the isolated cell is known to be susceptible to enhancement of its post-translational characteristic of interest by being contacted with a particular reagent, this reagent is used to obtain an enhancement of the mRNA signal in this cell, and/or the gene itself, which consequently produces amplified mRNA copies and thus ultimately cDNA copies, corresponding to that particular gene, or amplified gene segments. Both the cDNA and the genomic DNA genetic libraries may otherwise be obtained using known techniques. Once the genetic library is obtained, it is used to transform host cells using known techniques (e.g., by calcium phosphate precipitation, liposomal transfection, DEAE dextran transfection, microinjection, etc.).

Host cells useful in the present invention are preferably susceptible to lectin or antibody detection of the desired post-translational characteristic; that is, susceptible to

lectin or antibody detection of membrane-bound oligosaccharide, polysaccharide, or of glycoprotein or glycolipid produced in the transformed host cell. However, screening of host cells not susceptible to such lectin or antibody detection may be achieved through screening for enzyme activity in accordance with the invention.

(A) Host (e.g. mammalian) cells should be eukaryotic cells to allow and preserve the catalytic function of the enzyme (the glycosyltransferase). (B) The host cell should not express significant levels of glycosyltransferase activity analogous to the desired one, or the cognate product. With glycosyltransferase-related genes, successful transformation of a host cell can be determined by detecting corresponding enzymatic activity in an extract of the cell. (C) In another characteristic, the host cell should be capable of synthesizing the appropriate sugar nucleotide substrate and transport it into Golgi (where glycosyltransferase catalytic domains exist and function). Virtually all wild type animal cells possess this function. (D) The host cell should possess the ability to synthesize the appropriate acceptor substrate (oligosaccharide, lipid, or protein) that the desired glycosyltransferase requires, and the cell must display the structure on the cell surface or release it into the cellular environment/media. (E) The host cell should allow or provide for expression of transfected sequences that encode or otherwise determine expression of the desired



glycosyltransferase. This is inherent in eukaryotic (e.g. mammal) to eukaryotic (e.g. mammal) genomic DNA transfer, or in vector systems chosen for cDNA expression system, using known technology. (F) The host cell should allow for rescue of the transfected sequences that encode or otherwise determine expression of the relevant glycosyltransferase.

Wild-type eukaryotic (e.g. mammalian) cells possess these characteristics generally. Any particular wild-type cell of interest which does not possess criteria (B) or (D) set forth above, may be mutated, using standard techniques, to obtain a mutant cell possessing either of these criteria. If an enzyme assay-based selection method is used, then the criteria (C) and (D) set forth above are not necessary.

Once the host cells have been transformed, the population is screened for host cells containing the genetic material of interest. This is achieved by determining whether the host cell possesses the post-translational characteristic of interest, i.e., by detecting enzymatic activity in an extract of the transformed host cell, detecting membrane-bound oligosaccharide or polysaccharide on the cell, or detecting soluble oligosaccharide or polysaccharide in an extract of or secreted by the cell. The host cells which test positive are isolated, and the gene of interest can be retrieved from these transformed cells.

If the host cells are transformed by genomic DNA transfection, the gene rescue may be carried out as follows:

(a) molecular cloning by hybridization, via tagging of transfected genomic sequences by species-specific repetitive sequences; or

(b) tagging of transfected genomic sequences by in vitro or in vivo ligation to marker sequences.

If cDNA is used to transform the host cells, the gene/cDNA rescue may be carried out as follows:

(a) episomal rescue via Hirt procedure, or

(b) integrated copy rescue via plasmid tag.

Further detail with regard to gene rescue is provided in the accompanying examples.

Example of appropriate donor and host cells include the following:

(I) Human Blood Group H  $\alpha(1,2)$ fucosyl-transferase -  
(Ernst et al, J. Biol. Chem. 264:3436-3447, 1989; Rajan et al, J. Biol. Chem. 264:11158-11167, 1989; Larsen et al, Proc. Natl. Acad. Sci. USA, 87:6674-6678, 1990).

A.) L cell host-mouse species.

B.) Does not express  $\alpha(1,2)$ fucosyltransferase activity. Does not express  $Fu\alpha(1,2)Gal$  structures on cell surface.

C.) Does synthesize GDP-fucose, the sugar nucleotide substrate of  $\alpha(1,2)$ fucosyltransferase.

D.) Does synthesize  $Gal\beta(1,4)GlcNAc-R$  molecules that are acceptor substrates for the enzyme, and expresses them at cell surface.

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E.) Mouse cells are known to be able to express human genes.

F.) Mouse cells do not contain DNA sequences similar to human Alu repetitive DNA sequences. These Alu sequences (species-specific repetitive sequences) were used to identify and ultimately rescue the human gene from the mouse transfectant cell line.

(II) Mouse  $\alpha(1,3)$ galactosyltransferase - Larsen et al, Proc. Natl. Acad. Sci. USA, 86: 8227-8231, 1989.

A.) Kidney cell line expressing SV40 virus large T antigen - COS-1 cell line, monkey species.

B.) Does not express  $\alpha(1,3)$ galactosyltransferase activity. Does not express Gal $\alpha(1,3)$ Gal structures on cell surface.

C.) Does synthesize UDP-galactose, substrate of  $\alpha(1,3)$ galactosyltransferase.

D.) Does synthesize Gal $\beta(1,4)$ GlcNAc-R molecules that are acceptor substrates for the enzyme, and expresses them at cell surface.

E.) cDNA/COS-1 cell expression system for cDNA libraries - standard technology.

F.) cDNA/COS-1 cell expression system for cDNA libraries - standard technology.

(III) Human Lewis Blood Group  $\alpha(1,3/1,4)$ fucosyltransferase - J.F. Kukowska-Latallo et al, Genes and Development, vol. 4, (1990), pp. 1288-1303.

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A.) Kidney cell line expressing SV40 large T antigen COS-1 cell line, monkey.

B.) Does not express significant levels of  $\alpha(1,3)$ fucosyltransferase activity. Does not express cell surface  $\text{Gal}\beta(1,4)[\text{Fu}\alpha(1,3)]\text{GlcNAc-R}$  structure.

C.) Does synthesize GDP-fucose, substrate of  $\alpha(1,3)$ fucosyltransferase.

D.) Does synthesize  $\text{Gal}\beta(1,4)\text{GlcNAc-R}$  molecules that are acceptor substrates for the enzyme, and expresses them at cell surface.

E.) CDM 7/COS-1 cell expression system for cDNA libraries - standard technology.

F.) CDM 7/COS-1 cell expression system for cDNA libraries - standard technology.

In the latter stages of selection for this gene, criteria C and D were not necessary because pools of cDNA clones were screened by transfecting them into COS-1 cells, and then directly assaying extracts prepared from the transfected cells for  $\alpha(1,3)$ fucosyltransferase activity.

In one of its embodiments, the present invention provides a method for isolating a gene encoding a glycosyltransferase, and the gene thus isolated. This glycosyltransferase may be a fucosyltransferase, a sialyltransferase, a N-acetylglucosaminyltransferase, a galactosyltransferase, a N-acetylgalactosaminyltransferase, a mannosyltransferase, a

sulfotransferase, a glucosyltransferase, an acetylase, or another glycosyltransferase.

Individual glycosyltransferases are known to be particularly related to different types of sugars transferred by the enzyme (Beyer et al, "Glycosyltransferases and Their Use in Assessing Oligosaccharide Structure and Structure-Function Relationships" Adv. Enzymology (1982) 52: 23-175 - hereby incorporated by reference). Thus a particular kind of sugar linkage found on an oligosaccharide, glycoprotein, or glycolipid in or on a cell is associated with a particular glycosyltransferase. Methods are known for identifying such linkages (see Beyer et al, supra), and can be used in accordance with the present invention to isolate the gene encoding the corresponding glycosyltransferase.

Sialyltransferases, one of the glycosyltransferase provided by the present invention, are associated with the following sialic acid linkages: (1) Sia $\alpha$ 2 $\rightarrow$ 6Gal; (2) Sia $\alpha$ 2 $\rightarrow$ 3Gal; (3) Sia $\alpha$ 2 $\rightarrow$ 6GalNac; (4) Sia $\alpha$ 2 $\rightarrow$ 6GlcNac; (5) Sia $\alpha$ 2 $\rightarrow$ 8Sia; (6) Sia $\alpha$ 2 $\rightarrow$ 4Gal; and (7) Sia $\alpha$ 2 $\rightarrow$ 4GlcNac.

Fucosyltransferases, another type of glycosyltransferases provided by the present invention, are associated with the following linkages: (1) Fuca(1 $\rightarrow$ 2)Gal $\beta$ -; (2) Gal $\beta$ (1 $\rightarrow$ 3)[Fuca(1 $\rightarrow$ 4)]GlcNac $\beta$ -; (3) Gal $\beta$ (1 $\rightarrow$ 4)[Fuca(1 $\rightarrow$ 3)]GlcNac $\beta$ -; (4) Gal $\beta$ (1 $\rightarrow$ 4)[Fuca(1 $\rightarrow$ 3)]Glc; (5) -GlcNac $\beta$ (1 $\rightarrow$ 4)[Fuca(1 $\rightarrow$ 6)]GlcNac $\beta$ 1 $\rightarrow$ Asn; (6) -GlcNac $\beta$ (1 $\rightarrow$ 4)[Fuca(1 $\rightarrow$ 3)]GlcNac $\beta$ 1 $\rightarrow$ Asn; (7) Fuca(1 $\rightarrow$ 6)Gal $\beta$ -; (8)

Fuc $\alpha$ (1 $\rightarrow$ 3)Gal $\beta$ -; (a) Glc $\beta$ 1 $\rightarrow$ 3Fuc $\alpha$ 1 $\rightarrow$ O-Thr and Fuc $\alpha$ 1 $\rightarrow$ O-Thr/Ser; (10) Fuc $\alpha$ 1 $\rightarrow$ Ceramide; and (11) Fuc $\alpha$ 1 $\rightarrow$ 3Fuc.

N-Acetylglucosaminyltransferases, also provided by the invention, are associated with the following linkages: (1) GlcNAc $\beta$ 1 $\rightarrow$ 4GlcNAc; (2) GlcNAc $\beta$ 1 $\rightarrow$ Asn; (3) GlcNAc $\beta$ 1 $\rightarrow$ 2Man; (4) GlcNAc $\beta$ 1 $\rightarrow$ 4Man; (5) GlcNAc $\beta$ 1 $\rightarrow$ 6Man; (6) GlcNAc $\beta$ 1 $\rightarrow$ 3Man; (7) GlcNAc $\alpha$ 1 $\rightarrow$ 3Man; (8) GlcNAc $\beta$ 1 $\rightarrow$ 3Gal; (9) GlcNAc $\beta$ 1 $\rightarrow$ 4Gal; (10) GlcNAc $\beta$ 1 $\rightarrow$ 6Gal; (11) GlcNAc $\alpha$ 1 $\rightarrow$ 4Gal; (12) GlcNAc $\alpha$ 1 $\rightarrow$ 4GlcNAc; (13) GlcNAc $\beta$ 1 $\rightarrow$ 6GalNAc; (14) GlcNAc $\beta$ 1 $\rightarrow$ 3GalNAc; (15) GlcNAc $\beta$ 1 $\rightarrow$ 4GlcUA; (16) GlcNAc $\alpha$ 1 $\rightarrow$ 4GlcUA; (17) GlcNAc $\alpha$ 1 $\rightarrow$ 4IdUA.

Galactosyltransferases, also provided by the invention, are associated with the following linkages: (1) Gal $\beta$ 1 $\rightarrow$ 4Glc; (2) Gal $\beta$ 1 $\rightarrow$ 4GlcNAc; (3) Gal $\beta$ 1 $\rightarrow$ 3GlcNAc; (4) Gal $\beta$ 1 $\rightarrow$ 6GlcNAc; (5) Gal $\beta$ 1 $\rightarrow$ 3GalNAc; (6) Gal $\beta$ 1 $\rightarrow$ 6GalNAc; (7) Gal $\alpha$ 1 $\rightarrow$ 3GalNAc; (8) Gal $\alpha$ 1 $\rightarrow$ 3Gal; (9) Gal $\alpha$ 1 $\rightarrow$ 4Gal; (10) Gal $\beta$ 1 $\rightarrow$ 3Gal; (11) Gal $\beta$ 1 $\rightarrow$ 4Gal; (12) Gal $\beta$ 1 $\rightarrow$ 6Gal; (13) Gal $\beta$ 1 $\rightarrow$ 4xylose; (14) Gal $\beta$ 1 $\rightarrow$ 1'-sphingosine; (15) Gal $\beta$ 1 $\rightarrow$ 1'-ceramide; (16) Gal $\beta$ 1 $\rightarrow$ 3 diglyceride; (17) Gal $\beta$ 1 $\rightarrow$ O-hydroxylysine; and (18) Gal-S-cysteine.

N-Acetylgalactosaminyltransferases also provided by the invention are associated with the following linkages: (1) (GalNAc $\alpha$ 1 $\rightarrow$ 3)[(Fuc $\alpha$ 1 $\rightarrow$ 2)]Gal $\beta$ -; (2) GalNAc $\alpha$ 1 $\rightarrow$ Ser/Thr; (3) GalNAc $\beta$ 1 $\rightarrow$ 4Gal; (4) GalNAc $\beta$ 1 $\rightarrow$ 3Gal; (5) GalNAc $\alpha$ 1 $\rightarrow$ 3GalNAc; (6) (GalNAc $\beta$ 1 $\rightarrow$ 4GlcUA $\beta$ 1 $\rightarrow$ 3) $_n$ ; (7) (GalNAc $\beta$ 1 $\rightarrow$ 4IdUA $\alpha$ 1 $\rightarrow$ 3-) $_n$ ; (8) -Man $\beta$  $\rightarrow$ GalNAc $\rightarrow$ GlcNAc $\rightarrow$ Asn.

Other glycosyltransferases, also provided by the invention, are associated with the following linkages:

## To GalNAc

Gal $\beta$ 1-3GalNAc  
Gal $\beta$ 1-4GalNAc  
Gal $\alpha$ 1-3GalNAc  
GlcNAc $\beta$ 1-3GalNAc  
GlcNAc $\beta$ 1-6GalNAc  
GalNAc $\alpha$ 1-3GalNAc  
Sia $\alpha$ 2-3GalNAc  
Sia $\alpha$ 2-6GalNAc

## To Gal

Gal $\beta$ 1-3Gal  
Gal $\alpha$ 1-3Gal  
Fuc $\alpha$ 1-2Gal  
GlcNAc $\beta$ 1-3Gal  
GlcNAc $\beta$ 1-4Gal  
GlcNAc $\beta$ 1-6Gal  
GlcNAc $\alpha$ 1-4Gal  
GalNAc $\alpha$ 1-3Gal  
GalNAc $\beta$ 1-3Gal  
GalNAc $\beta$ 1-4Gal  
Sia $\alpha$ 2-3Gal  
Sia $\alpha$ 2-6Gal

## To Glc

Man $\alpha$ 1-6Glc  
Man $\alpha$ 1-4Glc

## To GlcNAc

Gal $\beta$ 1-4GlcNAc

Gal $\beta$ 1-3GlcNAc

Fuc $\alpha$ 1-3GlcNAc

Fuc $\alpha$ 1-4GlcNAc

Glc $\alpha$ 1-4GlcNAc

GlcNAc $\alpha$ 1-4GlcNAc

Sia $\alpha$ 2-4GlcNAc

To Sia

Sia $\alpha$ 2-8Sia

To Protein

GalNAc $\alpha$ 1-O-Ser/Thr

Still other glycosyltransferases provided by the invention include:  $\beta$ 1,3GlcNAc- $\beta$ 1,3glucuronyltransferase, glucuronic acid- $\beta$ 1,4-N-acetylglucosaminyltransferase, asparagine N-acetylglucosaminyltransferase, serine  $\beta$ -xylosyltransferase, xylose  $\beta$ 1,4-galactosyltransferase, galactose  $\beta$ 1,3-galactosyltransferase, galactose  $\beta$ 1,3-glucuronyltransferase, glucuronic acid  $\beta$ 1,4-N-acetyl-galactosaminyltransferase, N-acetylgalactosamine  $\beta$ 1,3-glucuronyltransferase, N-acetylgalactosamine-4-sulfotransferase, N-acetylgalactosamine-6-sulfotransferase, asparagine- $\beta$ N-acetylglucosaminyltransferase, serine/threonine- $\alpha$ N-acetylgalactosaminyltransferase, N-acetylglucosamine- $\beta$ 1,4-galactosaminyltransferase, galactose- $\beta$ 1,3-N-acetylglucosaminyltransferase, N-acetylglucosamine-6-sulfotransferase, galactose-6-sulfotransferase, glucuronic

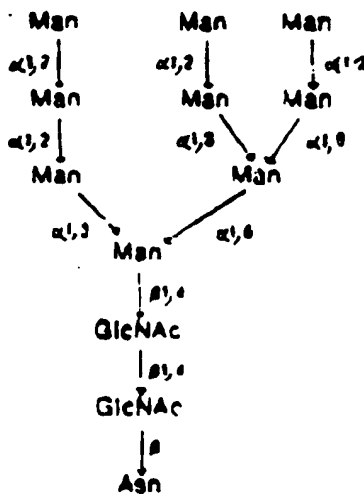


acid- $\alpha$ 1,4-N-acetylglucosaminyltransferase, N-acetylglucosamine  $\beta$ 1,4-glucuronyltransferase, heparin-N-acetyl-glucosamine-N-acetyltransferase, galactose-1,6-N-acetylgalactosyltransferase, heparin-N-acetylglucosamine sulfotransferase, N-acetylglucosamine- $\alpha$ 1,4-glucoronyl epimerase, N-acetylglucosamine-6-sulfotransferase, N-acetylglucosamine-N-sulfotransferase, glucuronyl- $\alpha$ 1,4-N-acetylglucosaminyltransferase, Iduronyl-2-sulfotransferase, glucuronyl- $\beta$ 1,4-N-acetylgalactosaminyltransferase, and N-acetylgalactosamine- $\alpha$ 1,3-glucuronyl epimerase.

These enzymes are associated with the following linkages and oligosaccharide structures in connective tissue polysaccharides (Roden, L. "Structure and Metabolism of Connective Tissue Proteoglycans," in The Biochemistry of Glyc2cograteoproteins and Proteoglycans, Wm. Lennarz, ed. pp. 267-371 Plenum Press, New York, incorporated herewith by reference, in particular the tables on pages 269, 270 and 271 thereof).

The above-noted glycosyltransferase genes and/or cDNAs are obtained as cloned molecules, or are transferred to host cell lines, in accordance with the invention by using the post-translational property manifest in the cognate and appropriate above-noted characteristic linkages, to isolate a cell from which the gene or cDNA library is then created, and from which the gene or cDNA encoding the glycosyltransferase is isolated.

Additional enzymes comprising members of the mannosyltransferase family include  $\alpha(1,2)$  mannosyltransferases,  $\alpha(1,3)$  mannosyltransferases,  $\alpha(1,6)$  mannosyltransferases, and  $\beta(1,4)$  mannosyltransferases, associated with the construction of the linkages formed in asparagine-linked oligosaccharides, as exemplified below (and as reviewed in Kornfeld, F., and Kornfeld, S. (1985) "Assembly of asparagine-linked oligosaccharides" Annu. Rev. Biochem. 54, pp. 631-664.)



Others include ceramide glucosyltransferase and ceramide galactosyltransferase, oligosaccharyltransferase, and O-acetylases that O-acetylate N-acetylneuraminic acid (sialic acid).

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Abbreviations:

Sia; sialic acid	IdUA: L-iduronic acid
Gal; D-galactose	GlcUA; D-glucuronic acid
GalNAc; D-N-acetylgalactosamine	Xyl; D-xylose
Glc; D-glucose	Ser; serine
GlcNAc; D-N-acetylglucosamine	Thr; Threonine
Fuc; L-fucose	Asn; asparagine--.
Man; D-mannose	

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In another embodiment, the present invention provides a method for obtaining a soluble or a solid-phase oligosaccharide, polysaccharide, lipid, or protein. This method comprises contacting an oligosaccharide or polysaccharide precursor with a fused protein. The enzyme used, provided by the present invention, is either an unglycosylated glycosyl transferase or a fused protein which comprises two moieties: as the first moiety, at least the catalytically functional domain of a glycosyltransferase (vide infra); and, as a second moiety, either a proteinaceous spacer attached to the solid support or a proteinaceous component comprising an affinity ligand. The enzyme of the invention

transforms the precursor into the desired oligosaccharide, polysaccharide, glycolipid, or glycoprotein which is thereby obtained.

A notable advantage of the invention is that it may provide, in one embodiment, non-glycosylated glycosyltransferases. It is thought to be generally true that many (if not all) naturally occurring glycosyltransferases are glycoproteins. When they are used to produce oligosaccharides or polysaccharides from oligosaccharide/polysaccharide precursors, the enzymes themselves may be susceptible to glycosylation. This (undesired) activity may consume starting material and may result in premature loss of enzyme activity. The non-glycosylated glycosyltransferases of the present invention do not suffer these salient disadvantages. They may be obtained as non-glycosylated enzyme either because they are obtained as a product produced in a microorganism deficient in the relevant glycosylation mechanism, or in an animal cell in which glycosylation of the glycosyltransferase has been suppressed. Suppression of the glycosylation of the glycosyltransferase in an animal cell is obtained by mutating the isolated glycosyltransferase gene using known techniques to eliminate the glycosylation sites on the glycosyltransferase.

The non-glycosylated glycosyltransferase of the present invention can be a non-glycosylated protein corresponding at least to the catalytically functional domain of the

glycosyltransferase (vide infra) and up to a non-glycosylated protein corresponding to the whole gene encoding the glycosyltransferase.

In another embodiment, the present invention provides a fused protein comprising two moieties as set forth above: as a first moiety, at least the catalytically functional domain of a glycosyltransferase; and, as a second moiety, either a proteinaceous spacer capable of being attached to a solid support or a proteinaceous component comprising an affinity ligand.

Glycosyltransferases are known to possess three domains which correspond to three different areas of the gene encoding the enzyme. The area of the gene found at the 3'-end of the gene is known to encode the catalytically functional domain (Lowe, Seminars in Cell Biology, (1991) 2:289-307 hereby incorporated by reference). The fused protein of the present invention contains at least this catalytically functional domain, but it can contain up to the whole protein sequence. The protein is produced fused to the second moiety using known techniques.

The second moiety can be used either to anchor the catalytically functional domain onto a solid support, or permit its recovery by exploiting the presence of a specific affinity ligand on the second moiety. For the second moiety, the IgG binding domain of Staph. protein A can be used. Such a fusion protein can be bound to an IgG-containing solid phase

matrix, such as IgG-Sepharose. A number of other alternative proteins can be fused to the catalytically active segments of the glycosyltransferase to effect binding to a solid matrix. Such proteins, and their respective matrix-associated receptors include streptavidin-biotin, IgG heavy chain-protein A, and virtually any other known protein or peptide segment for which an antibody exists or can be made.

In another embodiment, the present invention provides a method for producing a recombinant glycoprotein, glycolipid or free oligosaccharide using, e.g., either an enzyme obtained in accordance with the present invention or a recombinant organism obtained in accordance with the present invention. For example, specific post-translational glycosylation capability can be added to a host cell by the following steps: first the desired gene or cDNA which has been isolated is introduced into a cell by using standard transformation or transfection techniques in a manner to obtain an organism capable of expression of the transfected cloned gene product; the host cell acquires the post-translational capability determined by the transfected gene, where the cell did not express this capability prior to transfection. Alternatively, the approach set forth above is performed, but instead of using a single cloned gene that determines post-translational capabilities, use is made of uncloned gene segments (high molecular weight genomic DNA, for example) or a library of cloned genomic DNA fragments or cDNA molecules. Transfected

cells generated in this manner are then subjected to selection methods based upon detection of a newly-acquired desired post-translational capability, to isolate clonal cell lines expressing this capability.

In another embodiment, enzymes obtained in accordance with the present invention can be used in an in vitro reaction to modify cell-surface oligosaccharide molecules. For example, the inventor has purified the blood group A UDP-GalNAc transferase and its substrate UDP-GalNAc to convert in vitro blood group H oligosaccharide determinants on mouse cells to blood group A determinants (Ernst et al, J. Biol. Chem. (1989) 264:3436-3447). An analogous scheme can be employed using enzymes obtained in accordance with the present invention, alone, or in conjunction with other available glycosyltransferases and glycohydrolases, to modify existing cell surface oligosaccharide molecules on dead or viable, functional eukaryotic or prokaryotic cells, in such a manner as to render their cell surface oligosaccharide structures relevant for a desired characteristic.

Such a host cell possessing added specific post-translational glycosylation capability is used in accordance with known recombinant technology to obtain a glycoprotein, glycolipid, or free oligosaccharide. This cell being characterized by possessing both the post-translational glycosylation capability as well as the capability of

producing the recombinant glycoprotein, glycolipid, or free oligosaccharide.

These latter embodiments can be used, for example, to add novel oligosaccharide molecules to the surface of specific kinds of mammalian cells (cells with specific or general immune function, for example) for the purpose of targeting them to particular tissues or other locations in the body, for a therapeutic or diagnostic use. In particular, such modified cells could then be targeted to tissues expressing lectin-like cell adhesion molecules that specifically recognize particular oligosaccharide structures that have been added to the surface of the modified cells.

In another embodiment, the present invention provides a method for suppression of glycosylation activity in a cell. In this embodiment, specific post-translational glycosylation capability is deleted from a host cell. This result can be achieved by introducing a specific cloned reagent into a cell by standard transformation or transfection techniques, after in vitro modifications that (i) inactivate the gene and (ii) insert with it or adjacent to it one or more genetically selectable markers. Introduction of this modified inactive gene effectively replaces the endogenous functional gene via homologous recombination, using standard techniques.

If necessary, two or more rounds of this process can be performed to inactivate both wild type genes in a diploid (or higher ploidy) organism. The end result is a cell line with



(two) non-functional genes not now capable of determining the post-translational capability whose elimination was desired. Alternatively, the gene obtained in accordance with the present invention is introduced to a cell by transformation or transfection, in a state in which it is expressed in an anti-sense orientation, using standard techniques. This eliminates expression of its cognate wild type gene, via standard anti-sense expression methods. Treatment of the cell with anti-sense synthetic oligonucleotides, whose sequence(s) is (are) derived from the gene obtained in accordance with the present invention, can also be used to eliminate expression of the cognate wild type gene, again via standard methods.

Alternatively, the gene obtained in accordance with the present invention is introduced into a cell by transformation or transfection, such that the expression of a new post-translational modification prevents or eliminates expression of an undesired one. This approach turns on the observation that the actions of some glycosyltransferases on common acceptor substrates are mutually exclusive, i.e.,  $\alpha(1,2)$ fucosylation can prevent  $\alpha(2,3)$ sialylation and vice versa, or  $\alpha(1,3)$ galactosylation can prevent  $\alpha(2,3)$ sialylation, and vice versa.

Addition or deletion of cellular post-translational capabilities (including glycosylation) allows, for example, the generation of host cell lines that can be used to produce lipids, proteins, or free oligosaccharides of diagnostic or

therapeutic utility, whose specific post-translational modifications, including glycosylation, affect their function. For example, recombinant proteins such as tissue plasminogen activator or erythropoietin normally exists as glycoproteins. Should specific oligosaccharide structures on these glycoproteins be shown to have beneficial effects on their biosynthesis, serum half life, receptor interaction, or other function, the reagents and processes provided by the present invention can be used to construct hosts that yield recombinant proteins with the specific, and functionally optimal, oligosaccharide structures.

This embodiment can be used, for example, to delete specific oligosaccharide molecules from the surface of specific kinds of mammalian cells (cells with specific or general immune function, for example) for the purpose of preventing targeting to their normal, physiologic tissues or other locations in the body, and thus allow them to be targeted to other non-physiologic targets for therapeutic or diagnostic use. In particular, such modified cells can be shunted away from tissues where they normally act, to tissues expressing lectin-like cell adhesion molecules with specificities for other kinds of cells.

In another embodiment, the present invention provides gene products in heretofore unavailable amounts. These gene products, glycosyltransferase enzymes, can be used in enzymatic reactors to produce glycoproteins, glycolipids,

oligosaccharides or polysaccharides of interest. In this embodiment, cloned glycosyltransferase gene segments can be used with standard recombinant protein expression systems to generate large amounts of the enzyme encoded by the gene. These enzymes can be used in bioreactors in in vitro, large scale, synthesis of oligosaccharides or glycolipids, or for glycosidic modification of proteins and glycoproteins.

Acceptor oligosaccharides in such a scheme can be derived from any of the following:

(a) commercially available mono-, di- or higher order saccharides prepared from natural sources, or by chemical synthesis;

(b) di- or higher order oligosaccharides produced in vitro by other recombinant enzymes generated by this process;  
or

(c) di- or higher order oligosaccharides produced by or purified from cell lines whose post-translational capabilities have been engineered as described above.

In this embodiment, two in vitro bioreactor-type approaches can be used. In one embodiment, an oligosaccharide acceptor and nucleotide sugar substrate are introduced into the reactor containing a solid phase matrix to which is bound catalytically active glycosyltransferase. This matrix can be generated using the fusion protein noted above which comprises a catalytically active moiety, as a soluble segment of the glycosyltransferase, fused to a protein segment that can be

used to bind the fusion protein to a solid phase matrix. A specific example of such a fusion protein is a catalytically active segment of the mouse  $\alpha(1,3)$  galactosyltransferase, fused to a segment of the IgG binding domain of Staph. protein A (Larsen et al, Proc. Natl. Acad. Sci. (USA), 86, 8227-8231, 1989).

Acceptor and nucleotide sugar substrates are incubated in such a reactor, at an appropriate temperature, pH, and other known conditions, for a length of time sufficient to yield a desired amount of oligosaccharide or polysaccharide product. The product is then harvested by using known techniques.

In the variation, the nucleotide sugar substrate and soluble glycosyltransferase catalytic domain-containing fusion protein is introduced into a reactor containing the oligosaccharide acceptor molecule covalently attached (i.e., immobilized) to a solid phase matrix. Attachment is carried out using known techniques in such a manner as to make available to the reaction medium the portion of the oligosaccharide acceptor molecule that will be enzymatically modified.

The present invention provides a method for generating animal cell lines with specific capabilities for post-translational modification of proteins produced by them, as well as a method to isolate cloned genes, cloned complementary cDNAs, and their mRNAs, that determine the expression or biosynthesis of the enzymes responsible for post-translational

modification of eukaryotic (e.g. animal, such as mammalian) proteins, especially (but not limited to) those post-translational processes that construct eukaryotic (e.g. animal, such as mammalian) glycoconjugates, without the need to first isolate the protein products of these genes. This includes cloned genes, cloned complementary cDNAs, and their mRNAs, that encode eukaryotic (e.g. animal, such as mammalian) enzymes that post-translationally modify proteins by glycosylation and sulfation, as well as phosphorylation, methylation, fatty acylation, and removal of glycosyl modification (glycohydrolases).

The uses of the present invention thus include the following:

(i.) Construction of animal cell lines with specific post-translational capabilities (for the production of diagnostics and therapeutics).

This method can be used to construct animal cell lines that are suitable host cells for the production of diagnostic or therapeutic material whose usefulness or efficacy depends upon specific post-translational modifications. For example, the biological effectiveness of most therapeutic proteins or peptides, recombinant or otherwise, often depends critically upon the oligosaccharide structure(s) that are covalently attached to them. The structure of these oligosaccharides is primarily a function of the number and kind of

glycosyltransferase enzymes that are found in the cell used to produce these therapeutic products.

Animal cells and yeasts are competent to perform these glycosylation reactions; however, not all glycosyltransferase enzymes are produced by every animal cell or yeast, and therefore, some oligosaccharide structures (including sialyl Lewis X and/or sialyl Lewis a determinants, and/or their non-sialylated variants generated by the enzymes encoded by the present DNA sequences) are not produced by them. The converse is also true, namely, that the producing cell may express some glycosyltransferases that create oligosaccharide structures which prevent effective bioactivity. The present invention provides for the creation or elimination of specific glycosyltransferases capabilities in producing cells, so that therapeutic effectiveness of products made by the cells may be optimized.

It is likely that sialyl Lewis x and/or sialyl Lewis a determinants, and/or their non-sialylated variants, may alter the bioactivity (for better or for worse) of natural or recombinant therapeutic or diagnostic agents (glycoproteins or glycolipids) produced by mammalian or other eukaryotic hosts. Eukaryotic host cells that the biotechnology industry uses to produce these recombinant agents may be altered with the DNA sequence information and related information described in this invention, to add sialyl Lewis x and/or sialyl Lewis a determinants, and/or their non-sialylated variants, to the

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oligosaccharides on recombinant product by expressing one or more of the chimeric enzymes described here in the desired host.

The old methods used for this process include an empirical approach to identify a cell line most appropriate for the production of the recombinant or natural product. This is generally not optimal since cell lines with suitable post-translation modification capabilities may not exist naturally, or may not be especially suited to high level production of an appropriately modified product. Alternatively, unwanted post-translational modifications present on a therapeutic material produced by an empirically identified animal cell line can be removed chemically or enzymatically, a process that may be costly or inefficient, or both.

The advantages of the present methods over the older methods include the ability to construct cell lines with specific post-translational modification capabilities; properly constructed, these cell lines eliminate any need for chemical or enzymatic treatment of a therapeutic or diagnostic material to remove unwanted post-translational modifications. Moreover, cell lines with inappropriate post-translational modification capabilities, but that are otherwise excellent cells for production, may be modified to achieve correct post-translational modification of the product.

This method allows the construction of animal cell lines with post-translational modification capabilities precisely tailored to the specific needs of a particular diagnostic or therapeutic product produced by animal cells.

(ii.) Isolation of reagents suitable for efficient enzymatic synthesis and production of oligosaccharides (in enzyme reactors, for example).

Oligosaccharides can have therapeutic utility as immunomodulatory reagents in the field of organ transplantation. In particular, soluble and solid-phase oligosaccharides may find use as therapeutic agents with which to block or ameliorate antibody-mediated organ transplant rejection in cases involving incompatibility due to differences in the major blood group antigen systems of the organ donor and the recipient. Similarly, soluble oligosaccharides can find use as therapeutic agents that function by blocking attachment of bacterial, viral, or parasitic pathogens to glycoconjugate receptors found on the surface of the animal tissues that these pathogens invade. For example there is evidence that portions of the Lewis blood group oligosaccharide antigens serve as "receptors" for some forms of uropathogenic bacteria.

Moreover, glycoconjugates have been implicated in modulating adhesive events between cells and between cells and their environment during developmental and differentiation processes. These events included binding of spermatozoa to



eggs, and the initial events that mediate attachment of fertilized ova to the uterine wall at the beginning of implantation. These observations show, for example, the possibility that contraceptive uses for (biologically "natural") oligosaccharide molecules exist.

In addition, specific glycoconjugates containing sialyl Lewis x determinants have been implicated as ligands for the LECCAM/Selectin family of adhesion molecules, that play important roles in mediating adhesion between cells of the immune system, and some tumor cells, and the surfaces of the endothelial cells that line the vascular tree. These observations suggest the possibility that the cloned fucosyltransferase sequence described here could be used to construct oligosaccharide-type molecules, with pharmaceutical properties possessing anti-inflammatory and anti-tumor metastatic functions.

Currently, oligosaccharides of defined structure are produced by chemical synthesis (a procedure that is inefficient and costly) or by isolation from natural sources (using costly and inefficient procedures that often require the processing of large quantities of animal or plant material, and the purification of the desired oligosaccharide from other contaminating oligosaccharides) or by glycosyltransferase-assisted synthetic procedures, using other recombinant  $\alpha(1,3/1,4)$ fucosyltransferases.

The present invention provides a mechanism for the isolation of cloned glycosyltransferase genetic sequences, which in turn can be used to economically synthesize abundant quantities of purified glycosyltransferase enzymes. These can be used to construct enzyme bioreactors (enzymes in solution or immobilized on a solid phase matrix) capable of enzymatic synthesis of these structures.

This is more efficient than approaches involving the chemical synthesis of oligosaccharides or their purification from natural sources, for a variety of reasons. One, the only chemicals necessary are the enzyme substrates; most of these are easily obtained or synthesized. Two, enzymatic synthesis produces only the desired product and the nucleotide monophosphate or nucleotide diphosphate product of substrate hydrolysis. These latter two chemicals are found as the natural by-products of these reactions in animal cells, are essentially non-toxic, and may be easily separated from the oligosaccharide synthetic product.

By contrast, chemical synthetic procedures typically generate numerous products of side reactions which must be removed, and which may be toxic as well. Similarly, purification of oligosaccharides from natural sources requires the removal of other contaminating oligosaccharides present in the natural material.

Three, enzymatic catalysis is extraordinarily efficient; virtually complete conversion of substrate to product can be

achieved. By contrast, chemical synthesis of these structures is a multi-step process; yields at each step may be much much less than 100%, and the cumulative efficiency of current chemical synthesis procedures does not approach the efficiency possible with enzymatic synthesis. Similarly, purification of oligosaccharides from natural materials can entail significant losses inherent to the purification procedures required to separate the desired oligosaccharide from contaminating, irrelevant and/or undesirable oligosaccharides, with inefficient isolation of the desired oligosaccharide.

Although glycosyltransferases for synthetic use may be purified from animal tissues, these purifications are themselves inefficient, primarily because the enzymes are typically present in very low abundance. The present invention provides two mechanisms that provide for the abundant production of these enzymes.

First, this can be done through the construction and selection of animal cells that produce relatively large quantities of the enzymes. Alternatively, this invention provides a mechanism to isolate cloned cDNAs encoding these enzymes, or to construct synthetic genes that encode these enzymes via information derived from such cloned cDNAs or genes. These cloned nucleic acid sequences can then be used with standard recombinant DNA technologies to produce large quantities of glycosyltransferases.

(iii.) Isolation of reagents suitable for producing recombinant glycosyltransferases to be used directly as research reagents, or to be used to generate anti-glycosyltransferase antibodies for research applications.

The present invention provides two mechanisms for producing large quantities of these enzymes (see (ii.) above - i.e., specially constructed animal cells, or via natural or synthetic genes encoding these enzymes) which may be used as research tools with which to study the structures and functions of oligosaccharides and glycoproteins. Likewise, the enzymes produced by this method, or the nucleic acid sequence and derived protein sequence provided by this method, may be used to generate antibodies to these enzymes (via immunization with synthetic peptides whose sequences are derived from the cloned enzyme cDNAs or genes, or by direct immunization with the recombinant enzymes). These antibodies can also be used as research reagents to study the biosynthesis and processing of these enzymes, and can be used as an aid in their purification for all the uses described in this disclosure.

(iv.) Antibodies to glycosyltransferases as diagnostic reagents.

Some of these glycosyltransferases have been implicated as tumor markers in body fluids. The enzymes have typically been assayed in these fluids by activity assays, which may be subject to non-specificity due to competing

glycosyltransferase activity. These assays may also be insensitive since it is possible that inactive enzymes might be useful as tumor markers but would not be detected by enzyme activity assays.

The present invention provides a mechanism for generating antibodies to these enzymes (monoclonal and polyclonal antibodies against synthetic peptides constructed from information derived from cloned glycosyltransferase cDNAs or genes, against enzymes produced by recombinant glycosyltransferases, or against enzymes produced by animal cells constructed by this method). Anti-glycosyltransferase antibodies specific for particular glycosyltransferases can be produced by this means, and can be used to detect and quantitate glycosyltransferases in body fluids with specificity and sensitivity exceeding enzyme activity assays.

(v.) Engineering of glycosyltransferase substrate specificity to generate novel glycoconjugate structures on secreted or cell-associated glycoconjugates.

The present invention provides reagents (cloned glycosyltransferase genes or cDNAs) and genetic selection methods that, when used with appropriate known mutagenesis schemes, allow the generation of mutant glycosyltransferases that generate glycosidic linkages different from that generated by the wild-type enzyme. These novel linkages may or may not be naturally occurring, and find utility as moieties that enhance bioactivity of the molecules to which

they are attached. Alternatively, mutagenesis and selection approaches can be used to generate mutant enzymes that act in a dominant negative fashion. The dominant negative mutants so generated can be used to inactivate endogenous glycosyltransferase activities when the product(s) of such an enzyme are not desired.

This invention allows the isolation of glycosyltransferase genes (as well as genes that direct the synthesis of enzymes that perform the post-translational modifications) by methods designed to identify the surface-expressed product of the enzyme, and without the need to purify the enzyme as is required of standard molecular cloning procedures (i.e., without any information about the primary structure of the enzyme, and without antibodies directed against the enzyme).

A consequence of one implementation of this method is the generation of cells with specific capabilities for glycosylation. One version of the detailed implementation of this method is described in the following publications by the inventor, J. Biol. Chem. (1989) 264(6): 3436-3447 and J. Biol. Chem. (1989) 264(19): 11158-11167, both of which are herein incorporated by reference.

In outline, this version of the method entails the generation of cultured animal cell lines with specific abilities to construct desired glycoconjugate structures, by introducing exogenous genetic material into cells that do not

express the desired glycosyltransferase or its product, using genetic material from cells that do express the desired enzyme. A positive selection procedure is then employed to identify transfected cells that express the enzyme product on the surface of the cell. The transfected genetic sequences responsible for this new phenotype are then isolated by standard procedures involving gene library construction and nucleic acid hybridization. This method allows the isolation of the genetic material determining expression of the glycosyltransferase without the need to purify the enzyme.

Although detection and isolation of these sequences by hybridization procedures involving a dispersed and repetitive human DNA sequence (Alu) is used to illustrate isolation of the gene, other methods may be used to "tag" transfected sequences, including but not limited to the ligation to the transfected DNA of DNA markers that allow identification and isolation of the desired genes by nucleic acid hybridization or genetic selection (supF or G418 resistance "Neo" sequences, for example) procedures. Three methods for the selection of transfected cells with the appropriate phenotype, flow cytometry, "rosetting", and "panning", are described in Examples I, II, and III. Although an antibody specific for the enzyme product was used in the examples, other non-antibody reagents that specifically recognize surface expressed enzyme products may also be employed, including plant and animal lectins.

The enzymes provided by the present invention are imbued with certain unique characteristics, as compared to the corresponding native enzyme. Naturally-derived glycosyltransferases have been purified, with certain claims being made to the homogeneity of the product obtained. Nonetheless, such claims of homogeneity have been made based upon analyses of the preparations by SDS-polyacrylamide gel electrophoresis methods. In the older literature (i.e., pre-1982) the homogeneous enzyme was identified in the gel by Coomassie blue staining, or other staining methods, that are notably less sensitive than contemporary silver staining approaches. It is thus almost certain that such preparations were less than homogeneous.

In more contemporary literature, three glycosyltransferases have been analyzed by silver staining methods (i.e., rat sialyl-T, GlcNAc-T-I and GlcNAc-T-II). These appear to be virtually free of contaminant proteins. Nonetheless the small amounts of final pure proteins obtained using these purification procedures were analyzed using the sensitive silver staining method, which is not sufficiently sensitive to detect levels of contaminants of roughly 5 to 10 wt.%, in the small amounts of pure protein available. Thus, prior to the present invention, glycosyltransferases having a level of purity of at least 95 wt.%, preferably at least 98 wt.% were not available. The present recombinant glycosyltransferases which are obtained using cloned



glycosyltransferase DNA sequences, in large amounts, in soluble form, or fused to an affinity-purifiable protein segment, can be obtained in a truly, heretofore unavailable, homogeneous state.

The proteins provided by the present invention, as noted above, may also be distinguished from heretofore available proteins by the fact that they can be made in a non-glycosylated form. Many, if not all naturally-derived glycosyltransferases are glycoproteins, that is, they contain themselves one or more N-linked and/or O-linked oligosaccharide structures. These structures can themselves be glycosylated by the enzyme itself in an enzymatic reactor, for example, and this represents a competing acceptor substrate which could reduce the efficiency of the reaction and contribute to premature enzymatic activity loss. This "autoglycosylation" phenoma has the potential of either inactivating or reducing the catalytic efficiency of the enzyme and/or bioreactor.

Cloned glycosyltransferases provide a way to circumvent this problem. Firstly, expression of cloned glycosyltransferases in a bacteria host, such as E. coli, that is incapable of glycosylating these enzymes, will yield large amounts of non-glycosylated glycosyltransferases. These recombinant proteins can be used in a bioreactor, and since they are not themselves glycosylated, may be superior in

performance to the naturally derived, glycosylated enzymes for the reasons noted above.

Alternatively, if it is necessary to express these enzymes in an eukaryotic cell host that is capable of glycosylating the recombinant enzyme, standard site-directed mutagenesis approaches can be used to eliminate from the recombinant protein the amino acid signals that allow animal cells to glycosylate the enzymes. These known signals include certain asparagine residues, falling within the N-X-T or N-X-S motif that allows asparagine-linked glycosylation, and also includes some serine and threonine residues that are substrates for O-linked glycosylation.

Standard mutagenesis methods can be used to alter the DNA sequence encoding the glycosyltransferase to either delete the codon that encodes these N, S or T residues, or change the respective codon to a codon that determines an amino acid with similar physical properties, but that is incapable of supporting N-linked or O-linked glycosylation.

The present invention also provides unique mutant recombinant glycosyltransferases. Isolation and expression of glycosyltransferase genes and cDNAs offers the opportunity to generate mutant glycosyltransferases with properties superior to the fixed properties inherent in the naturally occurring enzymes. Standard techniques based upon site-directed, or random mutagenesis, can be used to obtain mutant glycosyltransferases with some of the illustrative properties:

(1) Minimal catalytic domain: progressive deletion of amino acids from the glycosyltransferase protein can be achieved, and the resulting mutant glycosyltransferases can be tested for activity. Based upon known functions for different parts of these molecules, it can be predicted that a catalytically active mutant glycosyltransferase can be produced that is (a) soluble (lacks transmembrane segment on natural glycosyltransferases that render them insoluble and thus unsuitable for bioreactors), and (b) much smaller than the natural glycosyltransferase (which retains transmembrane segment and the "stem" region, neither of which are necessary for catalytic activity).

On a protein mass basis, small catalytically-active domains derived from mutated glycosyltransferase genes or cDNAs represent more catalytic activity than the larger, naturally occurring glycosyltransferases that carry along non-catalytically active transmembrane and/or stem region protein "baggage." Thus, the recombinant mutant-derived catalytic domain is much more efficient for use in vitro synthesis of oligosaccharides, and by a reactor for example.

Approaches to amplification of mRNA for glycosyltransferases:

The cell line used as a source of genetic material (mRNA for cDNA library construction, or genomic DNA for genomic library construction or genomic DNA transfection) for gene transfer to isolate a glycosyltransferase gene can be

manipulated to optimize this process. Selection can be applied to this cell line to enhance steady state levels of the glycosyltransferase mRNA, and/or amplify its respective gene so that multiple copies of it exist in this donor cell. This can be done by subjecting the cell line (either after or without chemical, radiation, or other mutagenesis method) to a selection procedure that selects a variant of the cell that expresses higher amounts of the glycosyltransferase oligosaccharide product, at the surface of the cell, for example. This type of approach is illustrated in Example II.

Increased numbers of the oligosaccharide product molecules correlate with increased numbers of the cognate glycosyltransferase(s) enzyme molecules within the cell, and with an increase in steady state levels of the glycosyltransferase mRNA. Higher levels of this glycosyltransferase mRNA means that more copies of the respective cDNA will be present in a cDNA library prepared from the high-expression variant cell line, and thus will increase the likelihood of rescuing these glycosyltransferase cDNAs from the library. In some cases, higher levels of the specific mRNA can be associated with an increase in the number of copies of the cognate glycosyltransferase gene. Since such an amplified glycosyltransferase gene is more abundant in the cell's genome than other irrelevant genes, and more abundant than in a parental, non-selected cell line, it is easier to

isolate by genomic DNA library or genomic DNA transfection approaches.

It can be shown by transfection studies that expression of some oncogenes can increase expression of some glycosyltransferases. Thus a cell line can be modified by transfection with one or more oncogenes, using standard transfection methods, and readily available vectors containing oncogenes, and resultant transfected clones can be assayed for increased glycosyltransferase levels. Such clones can also be identified or selected for by FACS or lectin selection methods outlined in Examples I, II, and III below. These clones can then be used for cDNA library preparation as noted above.

A number of chemical reagents have been shown to induce expression of, or increase expression of, glycosyltransferases, in cell lines. This may be associated with in vitro differentiation of the cell line, and such agents include retinoic acid, as well as dimethylsulphoxide-induced differentiation of human and mouse hematopoietic precursors, with concomittant increases in the expression of some glycosyltransferases. This occurs because of an increase in the steady state level of mRNA for the glycosyltransferase in question, and can be used to enhance the ability to isolate a cognate cloned cDNA using the cDNA library-mediated transfection approach (as shown in Example II, below).

An alternative approach for the isolation of genes or cloned cDNAs that encode animal glycosyltransferases (or other

post-translational modification enzymes), by detecting the enzyme product at the cell surface, and without the need to purify the enzyme, is as follows: cDNA libraries are constructed in a plasmid or phage vector that will express the cloned cDNAs in a mammalian or yeast host, using mRNA prepared from cells or tissue that expressed the desired enzyme. This cDNA library is then screened for the desired cDNA by introducing the library into a host cell line that does not express significant amounts of the enzyme, nor its surface-expressed product, but that does have the necessary enzyme substrate molecules, and which is capable of displaying the enzyme's oligosaccharide product on its surface. The host cells which have taken up the cDNA library are subjected to selection for cells that contain the desired cDNA and thus express the new corresponding oligosaccharide product, using flow cytometry, rosetting, or panning, and a reagent specific for the enzyme's oligosaccharide product. Cloned cDNAs that direct the expression of the desired enzyme may then be isolated from the selected cells by standard methods.

This approach may be used with the following techniques:

1. Stable transfection into animal cells, selection, followed by rescue of the desired clone cDNA by nucleic acid hybridization procedures, or by the COS cell fusion technique, depending on the vector used.
2. Transient transfection into COS or WOP cells, selection, followed by rescue of the desired clone cDNAs, by

the method of Seed, Proc. Nat'l. Acad. Sci. (USA) (1987) 84:3365-3369, or similar methods that make use of cDNA cloning vectors that replicate as episomes in mammalian cells (i.e. Margolskee et al, Mol. Cell. Biol., (1988) 8:2837-2847).

3. Transformation of yeast cells, selection, followed by rescue of the desired cloned cDNA by nucleic acid hybridization procedures.

In addition, the mammalian cDNA expression libraries may also be screened by the sib selection method, using an enzyme assay to detect pools of cDNA molecules that contain cloned cDNAs encoding the relevant glycosyltransferase (or other enzyme involved in post-translational modification). Specifically, a cDNA library is constructed in a mammalian expression vector (plasmid or phage), using mRNA prepared from a cell that expresses the appropriate glycosyltransferase.

This library is then screened by first dividing it into pools of bacteria cells containing clones, each pool representing some fraction of the library, but the pools in aggregate representing the entire library. A portion of each pool is stored, and the remainder (containing sibs of the stored clones) is processed for cDNA-vector DNA molecules (i.e. plasmid or phage DNAs). The DNA prepared from each pool is separately introduced into one of the appropriate host cells described above (see 1, 2, and 3), and, after allowing for an appropriate expression time, extracts are prepared from

the transfected or transformed host cells and these extracts are assayed for the appropriate glycosyltransferase activity.

The smallest pool that is thus found to contain plasmids directing the synthesis of the appropriate enzyme is retrieved from storage and subdivided. Again, a representative portion of these pools is stored, and the remainder (again containing subs of the stored clones) of each is processed for plasmid DNA, transfection or transformation, expression, extract preparation, and enzyme assay. This process is repeated until single clones are isolated that direct expression of the relevant glycosyltransferase. Thus, this process does not rely upon surface expression of the enzyme product to isolate the appropriate cloned cDNA or gene. A version of this approach is presented in Example III.

The procedure used in the present invention need not be restricted by the genetic complement of the host; the gene transfer aspect of this invention allows expression of genes not normally expressed or even present in the recipient cell. Although the present text specifically illustrates application to glycosyltransferases, it may be applied to the enzymes and genes that control other forms of post-translational modification, including sulfation, phosphorylation, methylation, fatty acylation, and removal of glycosyl modification (glycohydrolases).

The method described to this point involves isolation of glycosyltransferase genes or cDNAs by selection for a dominant



glycosylation trait. The transient expression system described for use in COS or WOP cells can also be used to identify and clone cDNAs homologous to glycosyltransferase transcripts already present in the COS or WOP host.

Specifically, cloned cDNAs transcribed in the "anti-sense" orientation may eliminate expression of the cognate glycosyltransferase in the COS or WOP host, resulting in a recessive glycosylation trait. These DNA sequences can then be isolated by selection for surface expression of the oligosaccharide linkage recognized by the glycosyltransferase whose expression was eliminated, again by the procedures described below (flow cytometry, "rosetting", and "panning"), as detailed in Examples I, II, and III. Alternatively, the sib selection approach might be used to identify cloned cDNA molecules that decrease or eliminate the expression of an endogenous glycosyltransferase, as determined by enzyme assays.

The DNA sequences and corresponding glycosyltransferases of the present invention are summarized in the following Table.

TABLE 1

- A. Fuc-TIII (Lewis enzyme), SEQ ID NO:1 (DNA) and SEQ ID NO:2 (protein)  
 DNA at least nucleotide positions 199 through 1158 of SEQ ID NO:1, and up to the whole of SEQ ID NO:1  
 Protein at least amino acid positions 43 to 361 of SEQ ID NO:2, and up to the whole of SEQ ID NO:2
- B. Murine  $\alpha(1,3)$ galactosyltransferase, SEQ ID NO:3 (DNA) and SEQ ID NO:4 (protein)  
 DNA at least nucleotide positions 463 through 1461 of SEQ ID NO:3, and up to the whole of SEQ ID NO:3  
 Protein at least amino acid positions 63 to 394 of SEQ ID NO:4, and up to the whole of SEQ ID NO:4
- C. Human H  $\alpha(1,2)$  fucosyltransferase, SEQ ID NO:5 (DNA) and SEQ ID NO:6 (protein)  
 DNA at least nucleotide positions 4782 through 5783 of SEQ ID NO:5, and up to the whole of SEQ ID NO:5  
 Protein at least amino acid positions 33 to 3365 of SEQ ID NO:6, and up to the whole of SEQ ID NO:6
- D. Fuc-TIV, SEQ ID NO:7 (DNA) and SEQ ID NO:8 (protein)  
 DNA at least nucleotide positions 2089 through 3159 of SEQ ID NO:7, and up to the whole of SEQ ID NO:7  
 Protein at least amino acid positions 50 to 405 of SEQ ID NO:8, and up to the whole of SEQ ID NO:8
- E. Fuc-TV, SEQ ID NO:10 (DNA) and SEQ ID NO:11 (protein)  
 DNA at least nucleotide positions 247 through 1111 of SEQ ID NO:10, and up to the whole of SEQ ID NO:10  
 Protein at least amino acid positions 43 to 374 of SEQ ID NO:11, and up to the whole of SEQ ID NO:11
- F. Fuc-TVI, SEQ ID NO:13 (DNA) and SEQ ID NO:14 (protein)  
 DNA at least nucleotide positions 255 through 1208 of SEQ ID NO:13, and up to the whole of SEQ ID NO:13  
 Protein at least amino acid positions 43 to 359 of SEQ ID NO:14, and up to the whole of SEQ ID NO:14

SEQ ID NO:1 encodes a protein sequence termed Fuc-TIIII capable of functioning as a GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc)  $\alpha$ (1,3/1,4)-fucosyltransferase. This protein is an enzyme that can be used to construct the oligosaccharide "ligand" for Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1) that is disclosed in Applicant's co-pending U.S. Patent Application Serial No. 07/603,018, filed October 25, 1990, which is hereby incorporated by reference. This ligand is the sialyl-Lewis x molecule. Also, this enzyme, when expressed by the cloned DNA sequence described here, functions within mammalian cells to generate de novo expression of specific cell surface glycoconjugate structures on those cells. These structures are recognized by antibodies against the following cell surface glycoconjugate structures (See Fig. 8 and Table 2).

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SSEA-1 or Lewis x	Gal $\beta$ (1,4)[Fu $\alpha$ (1,3)]GlcNAc
sialyl-Lewis x	NeuAc $\alpha$ (2,3)Gal $\beta$ (1,4)[Fu $\alpha$ (1,3)]GlcNAc
Lewis a	Gal $\beta$ (1,3)[Fu $\alpha$ (1,4)]GlcNAc
sialyl-Lewis a	NeuAc $\alpha$ (2,3)Gal $\beta$ (1,3)[Fu $\alpha$ (1,4)]GlcNAc.

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In the above DNA sequence; (I), the sequence corresponding from amino acid position 43 to amino acid position 361 is functional, but a larger sequence of up to the whole sequence shown can be used.

This enzyme, when expressed by the cloned DNA sequence described here, functions in the enzymatic manner indicated in

its name, when assayed in extracts prepared from cells that express the DNA sequence (See Table 2). The oligosaccharide product of this enzyme represents fucose linked in alpha 1,3 configuration to neutral or  $\alpha(2,3)$  sialylated "type II" acceptors, or fucose linked in alpha 1,4 configuration to neutral or  $\alpha(2,3)$  sialylated "type I" acceptors as shown below:

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SSEA-1 or Lewis x	$\text{Gal}\beta(1,4)[\text{Fu}\alpha(1,3)]\text{GlcNAc}$
sialyl-Lewis x	$\text{NeuAc}\alpha(2,3)\text{Gal}\beta(1,4)[\text{Fu}\alpha(1,3)]\text{GlcNAc}$
Lewis y	$\text{Fu}\alpha(1,2)\text{Gal}\beta(1,4)[\text{Fu}\alpha(1,3)]\text{GlcNAc}$
Lewis a	$\text{Gal}\beta(1,3)[\text{Fu}\alpha(1,4)]\text{GlcNAc}$
sialyl-Lewis a	$\text{NeuAc}\alpha(2,3)\text{Gal}\beta(1,3)[\text{Fu}\alpha(1,4)]\text{GlcNAc}$
Lewis b	$\text{Fu}\alpha(1,2)\text{Gal}\beta(1,3)[\text{Fu}\alpha(1,4)]\text{GlcNAc}$

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Throughout the remainder of this text, these products will be referred to as sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues.

The catalytic domain of this enzyme has also been localized by expression studies. The enzymatic properties of the enzyme encoded by this cDNA, and chromosomal localization studies, indicate that this cDNA is the product of the human Lewis blood group locus.

This DNA sequence and the corresponding protein have the following uses:

(i.) Construction of animal cell lines with specific capabilities with respect to post-translational modification of the oligosaccharides on cell-surface, intracellular, or secreted proteins or lipids by sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$

fructose residues that represent the products of this enzyme (for the production of diagnostics and therapeutics).

Specifically, the present cloned DNA sequence can be introduced by standard technologies into a mammalian cell line that does not normally express the cognate enzyme or its product (sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fructose residues on oligosaccharides), and transcribed in that cell in the "sense" direction, to yield a cell line capable of expressing sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fructose residues on oligosaccharides on cell-surface, intracellular, or secreted glycoproteins or lipids.

Alternatively, this cloned DNA sequence may be introduced by standard technologies into a mammalian cell line that does express the cognate enzyme and its product (sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fructose residues), and transcribed in that cell in the "anti-sense" direction, to yield a cell line incapable of expressing sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fructose residues on cell-surface, intracellular, or secreted glycoproteins or lipids.

Alternatively, the endogenous GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc) $\alpha(1,3/1,4)$ -fucosyltransferase gene(s), in a mammalian cell expressing the cognate enzyme(s), can be inactivated with the DNA sequence described here by homologous recombination techniques, or by "anti-sense" gene expression or oligonucleotide approaches based upon the DNA sequence described herein, or by dominant negative mutant

fucosyltransferase sequences that inactivate endogenous GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc) $\alpha$ (1,3/1,4)-fucosyltransferase(s) and that may be derived via mutagenesis and genetic selection schemes, in conjunction with the sequence information in this text.

This method can be used to construct animal cell lines that are suitable host cells for the production of diagnostic or therapeutic materials whose usefulness or efficacy depends upon the specific post-translational modification determined by this cloned DNA sequence and its cognate enzyme. For example, it is known that the biological effectiveness of many therapeutic proteins or peptides, recombinant or otherwise, may depend critically upon the oligosaccharide structure(s) that are covalently attached to them. The structure of these oligosaccharides is primarily a function of the number and kind of glycosyltransferase enzymes that are found in the cell used to produce these therapeutic products.

Animal cells and yeasts are competent to perform these glycosylation reactions; however, not all glycosyltransferase enzymes are produced by every animal cell or yeast, and therefore, some oligosaccharide structures (including sub-terminal  $\alpha$ (1,3) and  $\alpha$ (1,4) fucose residues generated by the enzyme encoded by the DNA sequence described here) are not produced by them.

The converse is also true, namely, that producing cells may express a glycosyltransferase analagous to, or identical

to, the GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc) $\alpha$ (1,3/1,4)-Fucosyltransferase encoded by the DNA sequence described here. It is likely that sub-terminal  $\alpha$ (1,3) and  $\alpha$ (1,4) fucose residues alter the bioactivity (for better or for worse) of natural or recombinant therapeutic or diagnostic agents (glycoproteins or glycolipids) produced by mammalian or other eukaryotic hosts. Eukaryotic host cells that are used to produce these recombinant agents can be altered with the DNA sequence information and related information described in this invention, to add subterminal  $\alpha$ (1,3) and  $\alpha$ (1,4) fucose residues to the oligosaccharides on recombinant product by expressing all or part of the cloned sequences described here in the desired host. Alternatively, sub-terminal  $\alpha$ (1,3) and  $\alpha$ (1,4) fucose residues may be eliminated from the product produced in these host cells by the use of transfected "anti-sense" vector constructs, recombination-based gene inactivation, "anti-sense" oligonucleotide approaches, or dominant negative mutant fucosyltransferases, outlined above.

The old "methods" used for this process include an empirical approach to identify a cell line that does or does not express this particular enzyme or an enzyme that functions in a similar or identical manner, for the production of the appropriately modified recombinant or natural product. This is not always optimal since cell lines with this particular post-translation modification capabilities may not exist naturally, or may not be especially suited to high level

production of an appropriately modified product. Alternatively, unwanted sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues present on a therapeutic material produced by an empirically identified animal cell line must be removed chemically or enzymatically, a process that may be costly or inefficient.

The advantages of using the cloned, functional DNA sequence described here in conjunction with the technologies outlined above, relative to these older methods, include the ability to construct lines that specifically lack the capability to generate sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues on the oligosaccharides of glycoproteins and glycolipids; properly constructed, these cell lines will eliminate any need for chemical or enzymatic treatment of a therapeutic or diagnostic material to remove unwanted sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues. Moreover, in the event that sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues are found to be desirable for a particular diagnostic or therapeutic product produced by animal cells, cell lines may be engineered with the cloned DNA sequence described here to generate these residues.

(ii.) Isolation of reagents suitable for efficient enzymatic synthesis and production of oligosaccharides (in enzyme reactors, for example).

Oligosaccharides have therapeutic utility as immunomodulatory reagents in the field of organ



transplantation. In particular, soluble and solid-phase oligosaccharides find use as therapeutic agents with which to block or ameliorate antibody-mediated organ transplant rejection in cases involving incompatibility due to differences in the major blood group antigen systems of the organ donor and the recipient, including the Lewis blood group system. Likewise, soluble oligosaccharides may find use as therapeutic agents that function by blocking attachment of bacterial, viral, or parasitic pathogens to glycoconjugate "receptors" found on the surface of the animal tissues that these pathogens invade.

For example there is evidence that portions of the Lewis blood group oligosaccharide antigen (containing sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues) serve as "receptors" for some forms of uropathogenic bacteria. Moreover, glycoconjugates, including subterminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues, have been implicated in modulating adhesive events between cells, like leukocyte-ELAM-1 interactions, and between cells and their environment during developmental and differentiation processes. These events include binding of spermatozoa to eggs, and the initial events that mediate attachment of fertilized ova to the uterine wall at the beginning of implantation. These observations suggest, for example, the possibility that contraceptive uses for (biologically "natural") oligosaccharide molecules might exist. Oligosaccharide molecules constructed by this enzyme

can disrupt leukocyte-ELAM interactions and thus function as anti-inflammatory agents.

Currently, oligosaccharides containing subterminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues are produced by chemical synthesis (a procedure that is inefficient and costly or both) or by isolation from natural sources (using costly and inefficient procedures that often require the processing of large quantities of animal or plant material, and the purification of the desired oligosaccharide from other contaminating oligosaccharides).

The invention described here provides a mechanism to synthesize abundant quantities of purified GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc) $\alpha(1,3/1,4)$ -Fucosyltransferase, Fuc-TIII. This can be used to construct an enzyme bioreactor (enzyme in solution or immobilized on a solid phase matrix, for example via the protein-A moiety fused to the catalytic domain of the enzyme (as described in Kukowska-Latallo et al, Genes Devel., (1990) 4:1288-1303) capable of enzymatic synthesis of structures containing sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues.

This is more efficient than approaches involving chemical synthesis of structures containing sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues or their purification from natural sources, for a variety of reasons. One, the only chemicals necessary are the enzyme substrates and co-factors; these are easily obtained or synthesized. Two, enzymatic synthesis of

such structures will produce only the desired product and the nucleotide diphosphate product of substrate hydrolysis. This latter chemical is found as the natural by-product of these reactions in animal cells, is relatively non-toxic, and may be easily separated from the oligosaccharide synthetic product. By contrast, chemical synthetic procedures typically generate numerous products of side reactions which must be removed, and which may be toxic as well. Similarly, purification of oligosaccharides from natural sources requires the removal of other contaminating oligosaccharides present in the natural material.

Three, enzymatic catalysis is extraordinarily efficient; nearly or totally complete conversion of substrate to product might be achieved. By contrast, chemical synthesis of sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues on oligosaccharides is a multi-step process; yields at each step may be much less than 100%, and the cumulative efficiency of current chemical synthesis procedures does not approach the efficiency possible with enzymatic synthesis. Similarly, purification of oligosaccharides with sub-terminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose residues from natural materials can entail significant losses inherent to the purification procedures required to separate the desired oligosaccharide from contaminating, irrelevant oligosaccharides, with inefficient isolation of the desired oligosaccharide.

Although the GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc) $\alpha$ (1,3/1,4)-fucosyltransferase encoded by the DNA sequence described here may be isolated from animal tissues for synthetic use, these purifications are themselves inefficient, primarily because the enzyme is typically present in very low abundance.

This invention provides two mechanisms that provide for the abundant production of this enzyme. First, this may be done through the construction and selection of animal cells that produce relatively large quantities of the enzymes. Alternatively, this cloned nucleic acid sequence may be used with standard recombinant DNA technologies to produce large quantities of the fucosyltransferase in yeasts or in prokaryotic hosts. Furthermore, the sequence encoding this enzyme may be modified via standard molecular cloning schemes or mutagenesis to yield a recombinant fucosyltransferase with novel properties that make it more desirable than the wild-type enzyme.

For example, modifications can be made to the enzyme that make it more stable, or more suitable for immobilization in a bioreactor.

(iii.) Isolation of reagents suitable for producing recombinant GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc) $\alpha$ (1,3/1,4)-fucosyltransferase to be used directly as a research reagent, or to be used to generate antibodies against

the GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc)  $\alpha$ (1,3/1,4)-fucosyltransferase, for research applications.

This invention provides two mechanisms for producing large quantities of this enzyme (see ii. above - i.e., specially constructed animal cells, or via natural or synthetic genes encoding these enzymes) which may be used as a research tool with which to study the structures and functions of oligosaccharides and glycoproteins. Likewise, the enzyme produced by this method, or the nucleic acid sequence and derived protein sequence provided by this method, may be used to generate antibodies to this enzyme (via immunization with synthetic peptides whose sequences are derived from the cloned gene(s) or cDNA(s), or by immunization with the recombinant enzyme itself). These antibodies might also be used as research reagents to study the biosynthesis and processing of these enzymes, and can be used as an aid in their purification for all the uses described in this text.

(iv.) Antibodies to glycosyltransferases as diagnostic reagents.

Aberrant expression of GDP-Fuc:( $\beta$ -D-Gal(1,4/1,3)]-D-GlcNAc(/Glc)  $\alpha$ (1,3/1,4)-fucosyltransferase has been associated with malignancy in humans, suggesting that this enzyme might serve as a tumor marker for early detection of malignancy involving a number of human tissues. Enzyme tumor markers have typically been assayed in body fluids by activity assays, which may be subject to non-specificity due to

competing or similar glycosyltransferase activity. These assays may also be insensitive since it is possible that inactive enzymes might be useful as tumor markers but would not be detected by enzyme activity assays.

This invention provides a mechanism for generating antibodies to this enzyme (monoclonal and polyclonal antibodies against synthetic peptides constructed from information derived from cloned DNA sequence encoding GDP-Fuc:  $\beta$ -D-Gal(1,4/1,3)]-D-GlcNac(/Glc)  $\alpha$ (1,3/1,4)-fucosyltransferase, or against the recombinant enzyme produced by eukaryotic or prokaryotic hosts). Antibodies specific for this GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNac(/Glc)  $\alpha$ (1,3/1,4)-fucosyltransferase produced could be used to detect and quantitate this glycosyltransferase in body fluids, with specificity and sensitivity exceeding enzyme activity assays, and with the possibility of serving as a tumor marker for early detection of malignancy.

(v.) Recombinant enzyme for use in screening natural and synthetic compounds for fucosyltransferase inhibitors or inactivators.

A number of studies have noted an association between increased numbers of cell surface sub-terminal  $\alpha$ (1,3) and  $\alpha$ (1,4) fucose residues on oligosaccharides of a cell and the ability of that cell to metastasize in a malignant fashion. If there is a causal relationship, then drugs that inhibit the enzyme encoded by the sequence in this text could be active as

anti-tumor agents. Likewise, numerous recent studies have implicated sialylated and neutral oligosaccharides containing subterminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose linkages in mediating adhesion of leukocytes to the selectin adhesion molecules (ELAM-1; GMP-140; Mel14/LAM-1) during inflammation. These studies suggest that molecules capable of preventing synthesis of  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose linkages on leukocytes may thus function to diminish or even eliminate the ability of leukocytes to synthesize and display subterminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose linkages, and would thus represent anti-inflammatory pharmaceutical agents. The reagents described in this text are useful for screening to isolate compounds or identify compounds that exhibit antifucosyltransferase activity, since the cloned sequence may be used with standard techniques to produce relatively large amounts of pure fucosyltransferase. This further aids in screening since the effects of potential inhibitors will be tested on a pure enzyme, without the confounding effects that may occur in whole cell extracts or with partially purified enzyme.

(vi.) Engineering of glycosyltransferase substrate specificity to generate novel glycoconjugate structures on secreted or cell-associated glycoconjugates.

This invention provides a reagent a) cloned GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNac(/Glc)  $\alpha(1,3/1,4)$ -Fucosyltransferase cDNA) and the genetic selection method used to isolate it, that, when used with appropriate mutagenesis schemes, may

allow the generation of mutant GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNac(/Glc)  $\alpha$  (1,3/1,4)-Fucosyltransferases that generate glycosidic linkages different from that generated by the wild-type enzyme. These novel linkages may or may not be naturally occurring, and might find utility as moieties that enhance bioactivity of the molecules to which they are attached.

Alternatively, mutagenesis and selection approaches may be used to generate mutant GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNac(/Glc)  $\alpha$  (1,3/1,4)-Fucosyltransferases that act in a dominant negative fashion. The dominant negative mutants so generated can be used to inactivate endogenous glycosyltransferase activities when the product(s) of such an enzyme are not desired. Mutant GDP-Fuc:[ $\beta$ -D-Gal(1,4/1,3)]-D-GlcNac(/Glc)  $\alpha$  (1,3/1,4)-Fucosyltransferases can also be generated, for example, that function as fucosidases that hydrolyze various sugar linkages (fucose, mannose, or others) from oligosaccharides in vitro and in vivo.

(vii.) Genotyping individuals for the Lewis locus.

DNA sequence polymorphisms within or linked to the gene corresponding to this cloned cDNA may be used to genotype individuals for the Lewis locus. This can find utility with respect to organ transplantation procedures, or as a measure of susceptibility to infections caused by pathogens that may



use blood group structures as receptors for invasion (as in urinary tract infections, for example).

SEQ ID NO:3 encodes a mouse UDP-Gal: $\beta$ -D-Gal(1,4)-D-GlcNAc  $\alpha$ (1,3)-galactosyltransferase. SEQ ID NO:5 encodes a human GDP-Fuc: $\beta$ -D-galactoside  $\alpha$ (1,2)-fucosyltransferase. The uses for each of these proteins are generally the same as those discussed herein for the enzyme of SEQ ID NO:1.

Specific application of the enzyme encoded by SEQ ID NO:5 include enzymatic fucosylation of chain-terminating galactose residues on lactoseamine or neolacto type  $\beta$ -D-galactoside to  $\alpha$ -2-L-fucose residues. Such modification can be performed in vitro using the purified  $\alpha$ (1,2)FT, or its derivatives and its substrate GDP-fucose, using asialoglycans terminating with  $\beta$ -D-galactoside residues. Such asialoglycans exist naturally, and can also be constructed from glycans with terminal galactose moieties substituted with sialic acid by in vitro digestion with neuraminidase. Likewise, such fucosylation can be expected to occur when glycans are expressed in mammalian cells that have been transfected with the  $\alpha$ (1,2)FT cDNA or gene segment. Such  $\alpha$ (1,2) fucosylated glycans may have increased solubility properties, may have prolonged plasma half lives (by virtue of the fact that the terminal galactose residues that normally mediate glycoprotein clearance by the asialoglycoprotein receptor of the liver are now covered up by

fucose residues), differentiate from natural glycoforms, and may enhance bioactivity.

Molecular mechanisms used by cells to regulate the precise tissue-specific and developmental expression patterns of oligosaccharide structures are poorly understood. Such patterns however are probably determined largely by the coordinate regulation of expression of cognate glycosyltransferases. Since many of these enzymes recognize identical nucleotide sugar substrates or oligosaccharide acceptor substrates, it can be expected that they exhibit substantial primary protein and nucleic acid sequence similarities that would facilitate isolation of related glycosyltransferase genes by cross hybridization strategies.

Molecular cloning efforts by the inventor have allowed the isolation of several cloned glycosyltransferase cDNAs discussed above. Comparisons of the primary sequences of these enzymes reveals that they maintain virtually identical predicted structural topologies.

With the exception of one pair of distinct glycosyltransferases, however, there appear to be no substantial primary sequence similarities between these enzymes, even though many of them exhibit nucleotide sugar substrate or oligosaccharide acceptor substrate requirements that are virtually identical. The exceptional pair, a murine  $\alpha$ 1,3 galactosyltransferase sequence, or its human pseudogene

homologue, and a human  $\alpha 1,3N$ -acetylgalactosaminide transferase share substantial primary protein and nucleic acid sequence similarity, even though these enzymes use different nucleotide sugar substrates and exhibit distinct oligosaccharide acceptor substrate requirements.

Taken together, these observations indicate that some glycosyltransferases may be structurally related, but such relationships cannot necessarily be predicted from knowledge of nucleotide sugar or oligosaccharide acceptor substrate requirements.

As noted above, the gene transfer procedure of the invention has been used by the inventor to isolate a cloned cDNA that encodes the human Lewis blood group fucosyltransferase. This enzyme is an exceptional glycosyltransferase in that it can catalyze two distinct transglycosylation reactions. Sequence comparisons of this enzyme with the blood group H  $\alpha(1,2)$  fucosyltransferase indicates that these two fucosyltransferases maintain distinct primary sequences despite the fact that they use the identical nucleotide sugar substrate GDP-fucose, and can each utilize oligosaccharide acceptor molecules that terminate with unsubstituted type I or type II disaccharide moieties.

Biochemical and genetic data indicate that the human genome contains one or more structural genes that encode fucosyltransferases competent to construct surface localized SSEA-1 determinants. These enzymes are thought to be

polypeptides distinct from the Lewis fucosyltransferase because they exhibit different acceptor substrate specificities and differential sensitivities to divalent cation and N-ethylmaleimide inactivation. Moreover, their expression is determined by loci distinct from the Lewis blood group fucosyltransferase locus, and they display tissue specific patterns that are distinct from the Lewis locus patterns.

Because these enzymes exhibit properties that are very similar to the Lewis blood group fucosyltransferase, the inventor recognized that it was possible that these enzymes and their corresponding genes might be sufficiently related at the primary sequence level to be able to isolate them by crosshybridization approaches. In another embodiment, the invention therefore provides a method for isolating a gene encoding a glycosyltransferase by cross-hybridization. The cross-hybridization techniques which can be used in accordance with the invention are generally known. See, e.g., Lauer et al, Cell (1980) 20:119-130, Fritsch et al, Cell (1980) 19:959-972, Haynes et al, J. Biol. Chem. (1980) 255:6355-6367, and Proudfoot et al, Proc. Nat. Acad. Sci. (USA) (1979) 76:5425-5439, all of which are hereby incorporated by reference.

As noted above, oligosaccharides constructed by animal cells are remarkable for their structural diversity. This diversity is not random but rather consists of specific sets

of oligosaccharide structures that exhibit precise tissue-specific and developmental expression patterns. Molecular mechanisms used by cells to regulate these expression patterns are poorly understood. It can be expected, however, that such patterns are determined largely by the coordinate regulation of expression of the glycosyltransferases that determine these patterns. Recent molecular cloning efforts have allowed the isolation of several cloned glycosyltransferase cDNAs.

Comparisons of the primary sequences of these enzymes have revealed that they maintain virtually identical predicted structural topologies. With the exception of one pair of distinct glycosyltransferases, however, there appear to be no substantial primary sequence similarities between these enzymes, even though many of them exhibit nucleotide sugar substrate or oligosaccharide acceptor substrate requirements that are virtually identical. The exceptional pair, a murine  $\alpha 1,3$ galactosyltransferase sequence, or its human pseudogene homologue, and a human  $\alpha 1,3$ N-acetylgalactosaminide transferase share substantial primary protein and nucleic acid sequence similarity, even though these enzymes use different nucleotide sugar substrates and exhibit distinct oligosaccharide acceptor substrate requirements. Taken together, these observations suggest that some glycosyltransferases may be structurally related, but such relationships cannot necessarily be predicted from knowledge of nucleotide sugar or oligosaccharide acceptor substrate requirements. The inventor

has recently used a mammalian gene transfer procedure to isolate a cloned cDNA that encodes the human Lewis blood group fucosyltransferase (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990). This enzyme is an exceptional glycosyltransferase in that it can catalyze two distinct transglycosylation reactions. Sequence comparisons of this enzyme with the blood group H  $\alpha$ (1,2)fucosyltransferase (Larsen et al, Proc. Natl. Acad. Sci. USA, 87:6674-6678, 1990) indicates that these two fucosyltransferases maintain distinct primary sequences despite the fact that they use the identical nucleotide sugar substrate GDP-fucose, and can each utilize oligosaccharide acceptor molecules that terminate with unsubstituted type I or type II disaccharide moieties (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990; Larsen et al, Proc. Natl. Acad. Sci. USA, 87:6674-6678, 1990). Biochemical and genetic data indicate that the human genome contains one or more structural genes that encode fucosyltransferases competent to construct surface localized SSEA-1 determinants (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990; Potvin et al, J. Biol. Chem., 265:1615-1622, 1990). These enzymes are thought to be polypeptides distinct from the Lewis fucosyltransferase because they exhibit different acceptor substrate specificities and differential sensitivities to divalent cation and N-ethylmaleimide inactivation (Potvin et al, J. Biol. Chem., 265:1615-1622, 1990). Moreover, their expression is

determined by loci distinct from the Lewis blood group fucosyltransferase locus, and they display tissue specific patterns that are distinct from the Lewis locus patterns (Watkins, Adv. Hum. Genet., 10:1-116, 1980). Because these enzymes exhibit properties that are very similar to the Lewis blood group fucosyltransferase, the inventor considered it possible that enzymes and their corresponding genes might be sufficiently related at the primary sequence level to be able to isolate them by cross-hybridization approaches. The inventor has now achieved the isolation of several such cross-hybridizing human genes, an analysis of their structures, their expression in COS-1 cells after DEAE-dextran-mediated transfection, and analysis of their acceptor substrate properties.

Thus, in another embodiment, the invention provides DNA sequence SEQ ID NO:7 (set forth in Figure 4) that encodes a protein sequence capable of functioning as a GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferase, Fuc-TIV. This enzyme, when expressed by the cloned DNA sequence SEQ ID NO:7 functions within mammalian cells to generate de novo expression of specific cell surface glycoconjugate structures of those cells.

Present DNA sequence SEQ ID NO:9 (set forth in Figure 5 wherein it is identified as pFT-3 DNA) is comprised within DNA sequence SEQ ID NO:7. Namely, sequence SEQ ID NO:9 is

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found beginning at nucleotide position 1942 (the codon at nucleotide position 1942-1944) in sequence SEQ ID NO:7.

The DNA sequence and corresponding peptide provided in this embodiment of the invention must correspond at least to the segment from nucleotide position 2089 to 3159, preferably positions 1942 to 3156, of Sequence SEQ ID NO:7 set forth in Figure 4. These DNA sequences, can have further DNA sequence attached optionally to each end. These pendent DNA sequences can be of any length and up to a length corresponding to that set forth in Figure 4.

In a preferred embodiment, this embodiment of the invention provides DNA sequences, and their corresponding proteins, corresponding at least to the sequence between nucleotide positions 2089 to 3159, preferably positions 1942 to 3156, SEQ ID NO:7 and having attached to each end, optionally, further DNA sequences corresponding to those set forth in Figure 4. In this case, the pendent DNA sequences and corresponding proteins, can be of any length and up to the length set forth in Figure 4.

These glycoconjugate structures, constructed in part by this enzyme, are recognized by an antibody against the stage specific embryonic antigen I (SSEA-1 or Lewis x; structure  $\text{Gal}\beta(1,4)[\text{Fuc}\alpha(1,3)]\text{GlcNAc}$ ), and by an antibody against the VIM-2 determinant  $\text{NeuAc}\alpha(2,3)\text{Gal}\beta(1,4)\text{GlcNAc}\beta-(1,3)\text{Gal}\beta(1,4)[\text{Fuc}\alpha(1,3)]\text{GlcNAc}$ . This enzyme when expressed by DNA SEQ ID NO:7 functions in the enzymatic manner implied



in its name, when assayed in extracts prepared from cells that express the DNA sequence, as illustrated in Figure 8 and Table 2.

The oligosaccharide products of this enzyme represents fucose linked in alpha 1,3 configuration to the GlcNac residue of a "type II", lactos[amine] acceptor. Throughout the remainder of this disclosure, these products will be referred to as subterminal  $\alpha(1,3)$  fucose residues.

The isolation of three specific such cross-hybridizing human genes (SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:13), an analysis of their structure, their expression in COS-1 cells after DEAE-dextran-mediated transfection, and analysis of their acceptor substrate properties is described in the examples below, (Examples IV, V, and VI), and summarized in Figures 8 and Table 2. SEQ ID NO:10 (Fuc-TV) encodes a specific protein sequence capable of functioning as a GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNac  $\alpha(1,3)$  fucosyltransferase. This enzyme, when expressed by the cloned DNA sequence described, functions within mammalian cells to generate de novo expression of specific cell surface glycoconjugate structures on those cells. These structures are recognized by an antibody against the stage specific embryonic antigen I (SSEA-1 or Lewis x; structure Gal $\beta(1,4)$ [Fuc $\alpha(1,3)$ ]GlcNac), and by an antibody against the sialyl-Lewis x determinant NeuAc $\alpha(2,3)$ Gal $\beta(1,4)$  [Fuc $\alpha(1,3)$ ]GlcNac. This enzyme, when expressed by the cloned DNA sequence described, also functions

in the enzymatic manner implied in its name, when assayed in extracts prepared from cells that express the DNA sequence. The oligosaccharide products of this enzyme represent fucose linked in alpha 1,3 configuration to the GlcNac residue of a "type II" lactos[amine] acceptor. Throughout the remainder of this disclosure, these products will be referred to as subterminal  $\alpha(1,3)$  fucose residues. The location of the catalytic domain of this enzyme has been shown experimentally to encompass amino acids 43 to 374 of SEQ: ID NO:11.

The DNA and encoded protein of SEQ ID NO:13 and SEQ ID NO:14 (Fuc-TVI) may be used as follows:

- i. Construction of animal cell lines with specific capabilities with respect to post-translational modification of the oligosaccharides on cell-surface, intracellular, or secreted proteins or lipids by sub-terminal  $\alpha(1,3)$  fucose residues that represent the products of this enzyme (for the production of diagnostics and therapeutics by the biotechnology industry).

Specifically, the cloned DNA sequence described here may be introduced by standard technologies into a mammalian cell line that does not normally express the cognate enzyme or its product (sub-terminal  $\alpha(1,3)$  fucose residues on oligosaccharides), and transcribed in that cell in the "sense" direction, to yield a cell line capable of expressing sub-terminal  $\alpha(1,3)$  fucose residues on oligosaccharides on cell-surface, intracellular, or secreted

proteins or lipids. Alternatively, this cloned DNA sequence may be introduced by standard technologies into a mammalian cell line that does express the cognate enzyme and its product (subterminal  $\alpha(1,3)$  fucose residues), and transcribed in that cell in the "antisense" direction, to yield a cell line incapable of expressing sub-terminal  $\alpha(1,3)$  and fucose residues on cell-surface, intracellular, or secreted proteins or lipids. Alternatively, the endogenous GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha(1,3)$ -Fucosyltransferase gene(s), in a mammalian cell expressing the cognate enzyme(s), might be inactivated with the DNA sequence described here by homologous recombination techniques, or by "antisense" oligonucleotide approaches based upon the DNA sequence described herein, or by dominant negative mutant fucosyltransferase sequences that inactivate endogenous GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha(1,3)$ -Fucosyltransferase(s) and that may be derived via mutagenesis and genetic selection schemes, in conjunction with the sequence information in this Disclosure.

This method could be used to construct animal cell lines that will be suitable host cells for the production of diagnostic or therapeutic materials whose usefulness or efficacy depends upon the specific post-translational modification determined by this cloned DNA sequence and its cognate enzyme. For example, it is known that the biological effectiveness of many therapeutic proteins or

peptides, recombinant or otherwise, may depend critically upon the oligosaccharide structure(s) that are covalently attached to them. The structure of these oligosaccharides is primarily a function of the number and kind of glycosyltransferase enzymes that are found in the cell used to produce these therapeutic products. Animal cells and yeasts are competent to perform these glycosylation reactions; however, not all glycosyltransferase enzymes are produced by every animal cell or yeast, and therefore, some oligosaccharide structures (including sub-terminal  $\alpha(1,3)$  fucose residues generated by the enzyme encoded by the DNA sequence described here) are not produced by them. The converse is also true, namely, that producing cells may express a glycosyltransferase analogous to, or identical to, the GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha(1,3)$ -Fucosyltransferase encoded by the DNA sequence described here. It is likely that sub-terminal  $\alpha(1,3)$  fucose residues may alter the bioactivity (for better or for worse) of natural or recombinant therapeutic or diagnostic agents (glycoproteins or glycolipids) produced by mammalian or other eukaryotic hosts. Eukaryotic host cells that the biotechnology industry uses to produce these recombinant agents may be altered with the DNA sequence information and related information described in this invention, to add sub-terminal  $\alpha(1,3)$  fucose residues to the oligosaccharides on recombinant product by expressing all or part of the cloned sequences described here in the desired host. Alternatively,

sub-terminal  $\alpha(1,3)$  fucose residues may be eliminated from the product produced in these host cells by the use of transfected "anti-sense" vector constructs, recombination-based gene inactivation, 'anti-sense' oligonucleotide approaches, or dominant negative mutant fucosyltransferases, outlined above.

The old 'methods' used for this process include an empirical approach to identify a cell line that does or does not express this particular enzyme or an enzyme that functions in a similar or identical manner, for the production of the appropriately modified recombinant or natural product. This is not always optimal since cell lines with this particular post-translation modification capabilities may not exist naturally, or may not be especially suited to high level production of an appropriately modified product.

Alternatively, unwanted sub-terminal  $\alpha(1,3)$  fucose residues present on a therapeutic material produced by an empirically identified animal cell line must be removed chemically or enzymatically, a process that may be costly or inefficient. The advantages of using the cloned, functional DNA sequence described here in conjunction with the technologies outlined above, relative to these older methods, include the ability to construct lines that specifically lack the capability to generate sub-terminal  $\alpha(1,3)$  fucose residues on the oligosaccharides of glycoproteins and glycolipids; properly constructed, these cell lines will eliminate any need for chemical or enzymatic treatment of a therapeutic or diagnostic

material to remove unwanted sub-terminal  $\alpha(1,3)$  fucose residues. Moreover, in the event that sub-terminal  $\alpha(1,3)$  fucose residues are found to be desirable for a particular diagnostic or therapeutic product produced by animal cells, cell lines may be engineered with the cloned DNA sequence described here to generate these residues.

ii. Isolation of reagents suitable for efficient enzymatic synthesis and production of oligosaccharides (in enzyme reactors, for example).

Oligosaccharides may have therapeutic utility as immunomodulatory reagents in the field of organ transplantation. In particular, soluble and solid-phase oligosaccharides may find use as therapeutic agents with which to block or ameliorate antibody-mediated organ transplant rejection in cases involving incompatibility due to differences in the major blood group antigen systems of the organ donor and the recipient, including the Lewis blood group system. Likewise, soluble oligosaccharides may find use as therapeutic agents that function by blocking attachment of bacterial, viral, or parasitic pathogens to glycoconjugate "receptors" found on the surface of the animal tissues that these pathogens invade. For example there is evidence that portions of the Lewis blood group oligosaccharide antigen (containing sub-terminal  $\alpha(1,3)$  fucose residues) serve as "receptors" for some forms of uropathogenic bacteria.

Moreover, glycoconjugates, including sub-terminal  $\alpha(1,3)$  fucose residues, have been implicated in modulating adhesive events between cells, and between cells and their environment during developmental and differentiation processes. These events included binding of spermatozoa to eggs, and the initial events that mediate attachment of fertilized ova to the uterine wall at the beginning of implantation. These observations suggest, for example, the possibility that contraceptive uses for (biologically "natural") oligosaccharide molecules might exist. In addition, specific glycoconjugates containing sub-terminal  $\alpha(1,3)$  fucose residues have been implicated as ligands for the LECCAM/Selectin family of adhesion molecules, that play important roles in mediating adhesion between cells of the immune system, and some tumor cells, and the surfaces of the endothelial cells that line the vascular tree. Thus, the cloned fucosyltransferase sequence described here may be used to construct oligosaccharide-type molecules, with pharmaceutical properties possessing anti-inflammatory and anti-tumor metastatic functions.

Currently, oligosaccharides containing sub-terminal  $\alpha(1,3)$  fucose residues are produced by chemical synthesis (a procedure that is inefficient and costly) or by isolation from natural sources (using costly and inefficient procedures that often require the processing of large quantities of animal or plant material, and the purification of the desired oligosaccharide from other contaminating oligosaccharides).

The invention described here provides a mechanism to synthesize abundant quantities of purified GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferase. This could be used to construct an enzyme bioreactor (enzyme in solution or immobilized on a solid phase matrix, for example via the protein-A moiety fused to the catalytic domain of the enzyme, as described in previous manuscripts published by the Inventor John Lowe) capable of enzymatic synthesis of structures containing sub-terminal  $\alpha$ (1,3) fucose residues. This may be more efficient than approaches involving chemical synthesis of structures containing sub-terminal  $\alpha$ (1,3) fucose residues or their purification from natural sources, for a variety of reasons. One, the only chemicals necessary would be the enzyme substrates; these are easily obtained or synthesized. Two, enzymatic synthesis of such structures will produce only the desired product and the nucleotide diphosphate product of substrate hydrolysis. This latter chemical is found as the natural byproducts of these reactions in animal cells, is relatively non-toxic, and may be easily separated from the oligosaccharide synthetic product. By contrast, chemical synthetic procedures typically generate numerous products of side reactions which must be removed, and which may be toxic as well. Similarly, purification of oligosaccharides from natural sources requires the removal of other contaminating oligosaccharides present in the natural material. Three, enzymatic catalysis is extraordinarily



efficient; essentially complete conversion of substrate to product might be achieved. By contrast, chemical synthesis of sub-terminal  $\alpha(1,3)$  fucose residues on oligosaccharides is a multi-step process; yields at each step may be much less than 100%, and the cumulative efficiency of current chemical synthesis procedures does not approach the efficiency possible with enzymatic synthesis. Similarly, purification of oligosaccharides with subterminal  $\alpha(1,3)$  fucose residues from natural materials can entail significant losses inherent to the purification procedures required to separate the desired oligosaccharide from contaminating, irrelevant oligosaccharides, with inefficient isolation of the desired oligosaccharide. Although the GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha(1,3)$ -Fucosyltransferase encoded by the DNA sequence described here may be partially purified from animal tissues for synthetic use, these purifications are themselves inefficient, primarily because the enzyme is typically present in very low abundance. This invention provides two mechanisms that may provide for the abundant production of this enzyme. First, this may be done through the construction and selection of animal cells that produce relatively large quantities of the enzymes. Alternatively, this cloned nucleic acid sequence may then be used with standard recombinant DNA technologies to produce large quantities of glycosyltransferases in yeasts or in prokaryotic hosts. Furthermore, the sequence encoding this enzyme may be modified via standard molecular cloning schemes

or mutagenesis to yield a recombinant fucosyltransferase with novel properties that make it more desirable than the wild-type enzyme. For example, the modifications might be made to the enzyme that make it more stable, or more suitable for immobilization in a bioreactor.

iii. Isolation of reagents suitable for producing recombinant GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferase to be used directly as a research reagent, or to be used to generate antibodies against the GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferase, for research applications.

This invention provides two mechanisms for producing large quantities of this enzyme (see ii. above - i.e. specially constructed animal cells, or via natural or synthetic genes encoding these enzymes) which may be used as a research tool with which to study the structures and functions of oligosaccharides and glycoproteins. Likewise, the enzyme produced by this method, or the nucleic acid sequence and derived protein sequence provided by this method, may be used to generate antibodies to this enzyme (via synthetic peptides). These antibodies might also be used as research reagents to study the biosynthesis and processing of these enzymes, and might be used as an aid in their purification for all the uses described in this disclosure.

iv. Antibodies to glycosyltransferases as diagnostic reagents.

Aberrant expression of GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferase has been associated with malignancy in humans, suggesting that this enzyme might serve as a tumor marker for early detection of malignancy involving a number of human tissues. Enzyme tumor markers have typically been assayed in body fluids by activity assays, which may be subject to non-specificity due to competing glycosyltransferase activity. These assays may also be insensitive since it is possible that inactive enzymes might be useful as tumor markers but would not be detected by enzyme activity assays. This invention provides a mechanism for generating antibodies to this enzyme (monoclonal and polyclonal antibodies against synthetic peptides constructed from information derived from cloned DNA sequence encoding GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferase, or against the recombinant enzyme produced by eukaryotic or prokaryotic hosts). Antibodies specific for this GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferase so produced could be used to detect and quantitate this glycosyltransferase in body fluids, with specificity and sensitivity exceeding enzyme activity assays, and with the possibility of serving as a tumor marker for early detection of malignancy.

v. Recombinant enzyme for use in screening natural and synthetic compounds for fucosyltransferase inhibitors or inactivators.

A number of studies have noted an association between increased numbers of cell surface sub-terminal  $\alpha(1,3)$  fucose residues on oligosaccharides of a cell and the ability of that cell to metastasize in a malignant fashion. If there is a causal relationship here, then it may be possible that drugs that inhibit the enzyme encoded by the sequence in this disclosure might be active as anti-tumor agents. Likewise, numerous recent studies have implicated sialylated and neutral oligosaccharides containing subterminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose linkages in mediating adhesion of leukocytes to the selectin adhesion molecules (ELAM-1; GMP-140; Mel14/LAM-1) during inflammation. These studies suggest that molecules capable of preventing synthesis of  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose linkages on leukocytes may thus function to diminish or even eliminate the ability of leukocytes to synthesize and display subterminal  $\alpha(1,3)$  and  $\alpha(1,4)$  fucose linkages, and would thus represent anti-inflammatory pharmaceutical agents. The reagents described in this disclosure may prove useful for screening to isolate or identify compounds that exhibit anti-fucosyltransferase activity, since the cloned sequence may be used with standard techniques to produce relatively large amounts of pure fucosyltransferase. This will aid in screening since the effects of potential inhibitors will be

tested on a pure enzyme, without the confounding effects that may occur in whole cell extracts or with partially purified enzyme.

vi. Engineering of glycosyltransferase substrate specificity to generate novel glycoconjugate structures on secreted or cell-associated glycoconjugates.

This invention provides a reagent (a cloned GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferase gene segment), that, when used with appropriate mutagenesis and genetic selection schemes, may allow the generation of mutant GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferases that generate glycosidic linkages different from that generated by the wild-type enzyme. These novel linkages may or may not be naturally occurring, and could find utility as moieties that enhance the bioactivity of the molecules to which they are attached. Directed mutagenesis procedure may also be considered since this enzyme maintains primary sequence similarity to other  $\alpha$ (1,3)-Fucosyltransferases, yet exhibits a distinct set of acceptor substrate utilization properties. Alternatively, mutagenesis and selection approaches may be used to generate mutant GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferases that act in a dominant negative fashion. The dominant negative mutants so generated might be used to inactivate endogenous glycosyltransferase activities when the product(s)

of such an enzyme are not desired. Mutant GDP-Fuc:[ $\beta$ -D-Gal(1,4)]-D-GlcNAc  $\alpha$ (1,3)-Fucosyltransferases might also be generated, for example, that function as fucosidases that hydrolyze various sugar linkages (fucose, mannose, or others) from oligosaccharides in vitro and in vivo.

vii. Genotyping individuals at this fucosyltransferase locus.

Absence of a fucosyltransferase similar or identical to the one encoded by the DNA sequence detailed here has been found in several families. Should such absence be associated with a detrimental phenotype, DNA sequence polymorphisms within or linked to the gene corresponding to this cloned gene segment may be used to genotype individuals at this locus, for the purpose of genetic counseling. Likewise, the molecular basis for any such detrimental phenotypes might be elucidated via the study of the gene segment described here, should it be causally-related to such phenotypes.

In another embodiment, the present invention provides novel chimeric fucosyltransferases.

Five different human  $\alpha$ (1,3)fucosyltransferase ( $\alpha$ (1,3)Fuc-T) genes have been cloned. Their corresponding enzymes catalyze the formation of various  $\alpha$ (1,3)- and  $\alpha$ (1,4)-fucosylated cell surface oligosaccharides, including several that mediate leukocyte-endothelial cell adhesion during

inflammation. Inhibitors of such enzymes are predicted to operate as anti-inflammatory agents; in principle, the isolation or design of such agents may be facilitated by identifying peptide segment(s) within these enzymes that interact with their oligosaccharide acceptor substrates. Little is known, however, about the structural features of  $\alpha(1,3)$ Fuc-Ts that dictate acceptor substrate specificity. To begin to address this problem, we have created and functionally characterized a series of twenty-one recombinant  $\alpha(1,3)$ Fuc-T chimeras derived from three human  $\alpha(1,3)$ Fuc-Ts (Fuc-TIII, Fuc-TV, and Fuc-TVI) that maintain shared and distinct polypeptide domains and that exhibit common as well as idiosyncratic acceptor substrate specificities. The *in vivo* acceptor substrate specificities of these  $\alpha(1,3)$ Fuc-T chimeras, and of their wild type progenitors, were determined by characterizing the cell surface glycosylation phenotype determined by these enzymes, after expressing them in a mammalian cell line informative for the synthesis of four distinct  $\alpha(1,3)$ - and  $\alpha(1,4)$ -fucosylated cell surface oligosaccharides (Lewis x, sialyl Lewis x, Lewis a, and sialyl Lewis a). Our results indicate that as few as eleven non-identical amino acids, found within a "hypervariable" peptide segment positioned at the  $\text{NH}_2$ -terminus of the enzymes' sequence-constant COOH-terminal domains, determines whether or not these  $\alpha(1,3)$ Fuc-T can utilize type I acceptor substrates to form Lewis a and sialyl Lewis a moieties.

Cell surface  $\alpha(1,3)$ - and  $\alpha(1,4)$ -fucosylated oligosaccharides have received a substantial amount of attention because some are thought to be essential to the initiation of immune cell adhesion to vascular endothelium during the inflammatory process (Reviewed in Feizi, T. (1991) *TIBS*, vol. 16, 84-86; Springer, T.A., and Lasky, L.A. (1991) *Nature*, vol. 349, 196-197; Brandley, B.K., et al (1990) *Cell*, vol. 63, 861-863; Varki, A. (1994) *Proc. Natl. Acad. Sci. USA*, vol. 91, 7390-7397; Lowe, J. B., Carbohydrate recognition in cell-cell interaction. *Molecular Glycobiology*. In: *Frontiers in Molecular Biology*. Fukuda, M. (ed). Oxford University Press, Oxford, UK, 1994; Lowe, J.B., et al (1990) *Cell*, vol. 63, 475-484; Phillips, M.L., et al (1990) *Science*, vol. 250, 1130-1132; Walz, G., et al (1990) *Science*, vol. 250, 1132-1137; Goelz, S.E., et al (1990) *Cell*, vol. 63, 1349-1376; Tiemeyer, M., et al (1991) *Proc. Natl. Acad. Sci. USA*, vol. 88, 1138-1142; Larsen, E., et al (1990) *Cell*, vol. 63, 467-474; Polley, M.J., et al (1991) *Proc. Natl. Acad. Sci. USA*, vol. 88, 6224-6228; Zhou, Q., et al (1991) *J. Cell Biol.*, vol. 115, 557-564; Berg, E.L., et al (1991) *J. Biol. Chem.*, vol. 266, 14869-14872; Takada, A., et al (1991) *Biochem. Biophys. Res. Comm.*, vol. 179, 713-719). This is perhaps best characterized by the "rolling" type of adhesion mediated by interactions between sialyl Lewis x (sLe<sup>x</sup>)-bearing glycoconjugates on leukocytes and P- and E-selectin expressed by activated vascular endothelium (Lowe, J. B. Carbohydrate recognition in



cell-cell interaction. *Molecular Glycobiology*. In: *Frontiers in Molecular Biology*. Fukuda, M. (ed). Oxford University Press, Oxford, UK, 1994). Animal intervention studies indicate that such fucosylated oligosaccharide molecules can act as anti-inflammatory agents by inhibiting leukocyte-endothelial cell interactions (Takada, A., et al (1991) *Biochem. Biophys. Res. Comm.*, vol. 179, 713-719; Mulligan, M.S., et al (1993) *Nature*, vol. 364, 149-151; Mulligan, M.S., et al (1993) *J. Exp. Med.*, vol. 178, 623-631; Buerke, M., et al (1994) *J. Clin. Invest.*, vol. 93, 1140-1148). Furthermore, absence of such molecules on the leukocytes of individuals with the rare human leukocyte adhesion II syndrome is associated with profound defects in leukocyte-endothelial cell adhesion and with non-pyogenic infections (Etzioni, A., et al (1992) *New Engl. J. Med.* vol. 327, 1789-1792; von Adrian U.H., et al (1993) *J. Clin. Invest.*, vol. 91, 2893-2897). These observations suggest that compounds capable of specifically inhibiting leukocyte sialyl Lewis x expression might represent candidates for anti-inflammatory pharmacologic agents.

The  $\alpha(1,3)$ fucosyltransferases ( $\alpha(1,3)$ Fuc-Ts) represent a target for such inhibitory agents, since synthesis and expression of the sLe<sup>x</sup> molecule and related  $\alpha(1,3)$ - and  $\alpha(1,4)$ -fucosylated oligosaccharides are controlled, in large measure, by  $\alpha(1,3)$ fucosyltransferases ( $\alpha(1,3)$ Fuc-Ts) (Lowe, J. B. *Carbohydrate recognition in cell-cell interaction*. *Molecular Glycobiology*. In: *Frontiers in Molecular Biology*. Fukuda, M.

(ed). Oxford University Press, Oxford, UK, 1994). These enzymes catalyze the attachment of L-fucose in alpha anomeric linkage to one or more distinct oligosaccharide precursors. Biochemical and molecular cloning studies indicate that the human genome encodes at least five distinct  $\alpha(1,3)$ Fuc-Ts (Goelz, S.E., et al (1990) *Cell*, vol. 63, 1349-1376; Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303; Lowe, J.B., et al (1991) *J. Biol. Chem.*, vol. 266, 17467-17477; Kumar, R., et al (1991) *J. Biol. Chem.*, vol. 266, 21777-21783; Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584; Koszdin, K.L. and Bowen, B.R. (1992) *Biochem. Biophys. Res. Commun.*, vol. 187, 152-157; Sasaki, K., et al (1994) *J. Biol. Chem.*, vol. 269, 14730-14737; Natsuka, S., et al (1994) *J. Biol. Chem.*, vol. 269, 16789-16794; reviewed in Lowe, J. B. Carbohydrate recognition in cell-cell interaction. *Molecular Glycobiology*. In: *Frontiers in Molecular Biology*. Fukuda, M. (ed). Oxford University Press, Oxford, UK, 1994; Macher, B.A., et al (1991) *Glycobiology*, vol. 1, 577-584; Natsuka, S., and Lowe, J.B. (1994) *Curr. Opin. Struct. Biol.*, vol. 4, 683-691). Each enzyme can react with one or more structurally-distinct oligosaccharides and can thereby generate a unique spectra of cell surface  $\alpha(1,3)$ -fucosylated oligosaccharide products. In turn, these molecules may exhibit distinct biologic functions, including those involving selectin-dependent cell adhesion.

Although the primary sequences are known for several  $\alpha(1,3)$ Fuc-Ts (Goelz, S.E., et al (1990) *Cell*, vol. 63, 1349-1376; Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303; Lowe, J.B., et al (1991) *J. Biol. Chem.*, vol. 266, 17467-17477; Kumar, R., et al (1991) *J. Biol. Chem.*, vol. 266, 21777-21783; Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584; Koszdin, K.L., and Bowen, B.R. (1992) *Biochem. Biophys. Res. Commun.*, vol. 187, 152-157; Sasaki, K., et al (1994) *J. Biol. Chem.*, vol. 269, 14730-14737; Natsuka, S., et al (1994) *J. Biol. Chem.*, vol. 269, 16789-16794), the structural determinants within these enzymes that dictate their different substrate specificities remain undefined. The purpose of this work is to identify such protein sequence(s), and to provide a conceptual background for work to design or identify molecules that might inhibit  $\alpha(1,3)$ Fuc-Ts by interacting with acceptor substrate binding sites.

In this study, we chose to explore three human  $\alpha(1,3)$ Fuc-Ts (Fuc-TIII, Fuc-TV, and Fuc-TVI) with informative structural and catalytic properties. These enzymes share approximately eighty-five percent overall amino acid sequence identity (Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584). The COOH-termini of these enzymes maintain nearly identical amino acid sequences, while their NH<sub>2</sub>-terminal

regions are punctuated by foci of non-identical amino acids; we term these regions "hypervariable" segments. These three enzymes maintain shared and distinct acceptor substrate specificities. In particular, each of the three can effectively utilize neutral (Gal $\beta$ 1 $\rightarrow$ 4GlcNAc-) and  $\alpha$ (2,3)sialylated (NeuNAc $\alpha$ 2 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4GlcNAc-) type II acceptors to create cell surface Lewis x (Le<sup>x</sup>) and sLe<sup>x</sup> determinants, respectively, when tested by transfection in cultured cells (Weston, B.W., et al (1992) *J. Biol. Chem.*, vol, 267, 4152-4160; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584), and when tested with low molecular weight acceptor substrates in *in vitro* fucosyltransferase assays. Fuc-TIII can also efficiently utilize neutral (Gal $\beta$ 1-3GlcNAc) and sialylated (NeuNAc $\alpha$ 2-3Gal $\beta$ 1-3GlcNAc-) type I acceptor substrates to generate Lewis a (Le<sup>a</sup>) and sialyl Lewis a (sLe<sup>a</sup>) molecules (Lowe, J.B., et al 1990) *Cell*, vol. 63, 475-484; Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303) in transfected cells, and *in vitro*. By contrast, neither Fuc-TV nor Fuc-TVI directs formation of fucosylated type I antigens (i.e., Le<sup>a</sup> and sLe<sup>a</sup>) when tested in transfected cells, and neither can efficiently utilize low molecular weight acceptor substrates *in vitro* (Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584). Thus, the three enzymes' shared, and distinct, catalytic specificities, along with similarities in, and differences between, their

primary sequences, provide an opportunity to identify amino acid residues present in Fuc-TIII and/or absent from Fuc-TV and Fuc-TVI, that determine type I oligosaccharide acceptor substrate utilization.

To identify such residues, we studied the pair of enzymes that differ most dramatically in their respective abilities to utilize type I acceptors (Fuc-TIII versus Fuc-TVI; Figure 9a. We divided the amino-terminal "hypervariable" regions of each enzyme into five subdomains, each consisting of a distinct region of non-identical peptide sequence, separated by a region of identity (Fig. 9b). We then created a series of recombinant  $\alpha(1,3)$ Fuc-T chimeras in which these domains, or other domains derived from the enzymes' COOH-terminal regions, were transposed by themselves, or in combination, between the three enzymes, at corresponding positions. Functional analysis of these Fuc-TIII:Fuc-TVI chimeras (Fuc-TC's), and their wild type progenitors, via expression in a transfected mammalian cell line, indicates that the acceptor substrate specificity of these enzymes is determined by peptide sequences in the "hypervariable" NH<sub>2</sub>-terminal segments of these enzymes. When considered together with results obtained with a Fuc-TIII:Fuc-TV chimera, these analyses indicate that type I acceptor substrate utilization is determined by as few as eleven residues within a 61 amino acid long segment immediately proximal to the enzymes' "sequence-constant" COOH-terminal domains.

The present chimeric enzymes can be used in the same way as the other fucosyltransferases described herein.

Other features of this invention will become apparent in the course of the following description of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

#### EXAMPLES

**Example I.      Cloning and Expression of a DNA Sequence  
Encoding  $\alpha(1,2)$ Fucosyltransferase (DNA SEQ ID  
NO:5, protein SEQ ID NO:6**

Mouse L Cells as a Host for Human ( $\alpha$  - 1,2)Fucosyltransferase Gene Transfer - Mouse L cells were tested as a host for gene transfer. These cells have been widely used for this purpose. Genomic DNA may be introduced into L cells with high efficiency, and these cells allow the use of several metabolic and antibiotic resistance schemes for selecting stable incorporation of exogenous DNA sequences.

L cells were examined for surface expression of the H Fuc  $\alpha(1,2)$ Gal linkage, using a monoclonal antibody that recognizes type II H structures and fluorescence-activated cell sorting. Cells stained with this anti-H antibody exhibit a FACS profile virtually identical to the profile generated by cells stained with a control mouse IgM monoclonal antibody, and to profiles generated by cells stained only with

FITC-conjugated second antibody. These results indicate that L cells do not express surface-localized Fuc  $\alpha(1,2)$ Gal linkages that are detectable with the anti-H-antibody.

The inventor assayed L cell extracts to confirm that this absence of surface-expressed H determinants was due to a deficiency of  $(\alpha-1,2)$ fucosyltransferase activity.

Phenyl- $\beta$ -D-galactoside was used as the acceptor for assay of  $(\alpha-1,2)$ fucosyltransferase. This compound is a specific acceptor for  $(\alpha-1,2)$ fucosyltransferases; it is used efficiently by these enzymes, yet does not function as an acceptor for fucosyltransferases that generate  $(\alpha-1,3)$ ,  $(\alpha-1,4)$ , or  $(\alpha-1,6)$  linkages. L cell extracts contained no detectable  $(\alpha-1,2)$ fucosyltransferase activity, even in assays that contained increased amounts of extract, or that were subjected to prolonged incubation. Mixing experiments with A431 cell extracts showed that inhibitors were not responsible for an apparent lack of detectable enzyme activity.

The inventor also examined L cells for surface expression of glyconjugates possessing N-acetyllactosamine ( $\text{Gal}\beta(1,4)\text{GlcNAc}$ ) end groups. These would represent potential acceptor molecules for a complementing human  $(\alpha-1,2)$ fucosyltransferase activity and would allow the resulting surface-expressed  $\text{Fu}\alpha(1,2)\text{Gal}$  linkages to be detected with anti-H antibody. The agglutinin from *Erythrina cristagalli* (ECA) was used for this analysis. This lectin exhibits high affinity for oligosaccharides possessing one or

more unsubstituted N-acetyllactosamine end groups. L cells were stained with purified, FITC-labeled ECA, or with FITC-labeled ECA that had been preincubated with the hapten N-acetyllactosamine and were subjected to FACS analysis. The results indicated that significant amounts of ECA bind to these cells, and that the binding is effectively inhibited by the hapten N-acetyllactosamine. These results are consistent with the expectation that L cells synthesize oligosaccharides containing N-acetyllactosamine moieties and suggest that some of these glycoconjugates remain unmodified and are expressed at the cell surface.

The inventor also tested L cells for the ability to synthesize the fucosyltransferase substrate GDP-fucose. These analyses identified both GDP-[<sup>3</sup>H]fucose and GDP-[<sup>3</sup>H]mannose in aqueous extracts prepared from cells labeled with [2-<sup>3</sup>H]mannose. The subcellular location of GDP-[<sup>3</sup>H]fucose in these cells cannot be determined from these experiments. Effective Golgi fucosyltransferase activity would presumably require the presence of substrate concentrations of GDP-fucose within the lumen of the Golgi. Since these cells have not been selected to be defective in fucose metabolism, it seemed likely that they would be competent to transport this cytoplasmically synthesized compound into the Golgi lumen. This was confirmed by demonstrating that these cells are able to incorporate radio-labeled fucose into membrane glycoconjugates; most of this may represent fucose in  $\alpha(1,6)$



linkage to the asparagine-linked N-acetylglucosamine of some N-linked oligosaccharides.

Taken together, these studies show that L cells are competent to display surface-localized H  $\text{Fu}\alpha(1,2)\text{Gal}$  linkages, after introduction and expression of human DNA sequences determining  $(\alpha-1,2)$ fucosyltransferase activity.

**Human A431 Cells as a Donor for  $(\alpha-1,2)$ Fucosyltransferase DNA Sequences** - The human A431 cell line was investigated as a source of DNA for gene transfer since these cells express type I and II blood group H structures. Extracts prepared from A431 cells are found to contain  $(\alpha-1,2)$ fucosyltransferase activity when assayed using phenyl- $\beta$ -D-galactoside. The radiolabeled product elaborated by A431 extracts cochromatographed with authentic [ $^{14}\text{C}$ ] fucosylated phenyl- $\beta$ -D-galactoside produced by human serum H  $(\alpha-1,2)$ fucosyltransferase. Digestion of the A431 product with  $\alpha$ -L-fucosidase generated L-fucose in quantitative yield. These results indicate that A431 cells contain one or more functional  $(\alpha-1,2)$ fucosyltransferase genes and thus represent an appropriate source of human DNA for gene transfer.

**Isolation of a Mouse Transfectant That Expresses Surface Molecules Recognized by a Monoclonal Anti-H Antibody** - To isolate mouse cells containing DNA sequences that determine expression of a human  $(\alpha-1,2)$ fucosyltransferase, monolayer cultures of L cells were cotransfected with 30:1 ratio of high molecular weight genomic DNA prepared from A431 cells and

pSV2-neo plasmid DNA. Cotransfection with pSV2-neo followed by growth of the transfected cells in media containing G418 allows selection of transfectants that have stably incorporated exogenous DNA sequences. With this procedure the inventor generated a population of cells representing approximately 60,000 independent G418-resistant transfectants. This method typically incorporates approximately 1000 kb of transfected sequences into the genome of a recipient cell. Since the size of the human genome is approximately  $3 \times 10^6$  kb, the inventor estimated that approximately 20 copies of the haploid human genome were represented within this "library" of primary transfectants.

Transfectants were selected for H antigen expression by a combination of panning and sterile cell sorting. A pool of cells representing the entire population of transfected cells was reacted with a mouse IgM monoclonal antibody that recognizes type II H structures. Transfectants with bound anti-H antibody were subjected to an initial selection by panning on sterile dishes coated with goat anti-mouse IgM. At this stage, the inventor found this procedure to be more effective than selection by flow cytometry because it allowed larger numbers of transfectants to be rapidly and easily processed. Transfectants selected by panning were returned to culture to be amplified for subsequent rounds of selection. The FACS profile of cells present after this first selection revealed no obvious peak of cells that had bound the anti-H

antibody. However, analysis of the FACS histogram indicated that approximately 0.13% of the cells stained more brightly than cells stained with the control antibody. Cells representing the brightest 3-5% of the total population were aseptically collected, returned to culture for 14 days, and then reselected by the same procedure. After three selections, FACS analysis revealed the presence of a distinct population of cells that were brightly stained with the anti-H antibody. These cells were collected and returned to culture. For heuristic reasons, transfectants were also subjected to selection by panning, in parallel with the FACS selections. The inventor found that the panning procedure more efficiently enriched for populations of cells that bound the anti-H antibody. This is perhaps because the IgM anti-H antibody induced agglutination of H-positive transfectants and interfered with selection by FACS.

Therefore, all subsequent selections were performed by the panning procedure. After three additional rounds of panning (representing a total of seven rounds of selection), more than 90% of the cells within the selected population stained brightly with anti-H antibody. Clonal isolates from this population were generated, and individual subclones were analyzed for H antigen expression by FACS. Most clones gave rise to phenotypically mixed populations of cells consisting of H-expressing and non-expressing transfectants. The reasons for this apparent phenotypic instability are not known. One

clone that exhibited a stable, bright H antigen-positive phenotype was selected for further analysis (clone mH1-12). The phenotype of clone mH1-12 has remained stable for more than 9 months in the absence of selection of H expression.

The inventor wished to rule out the possibility that a murine ( $\alpha$ -1,2)fucosyltransferase gene might be active in rare variants in the L cell population or that the transfection procedure itself might activate this gene and that the selection process might enrich for these undesired events. Therefore, in a parallel control experiment, L cells were transfected with high molecular weight genomic DNA prepared from L cells, using pSV2-neo as the selectable marker. These transfectants were then subjected to selection for H antigen expression, exactly as described above. The inventor was unable to detect or isolate H-expressing cells from a population of independent transfectants (at least 40,000) that together has integrated the equivalent of more than 15 copies of the haploid murine genome.

The Primary Transfectant Expresses Cell Surface Type II Blood Group H Antigen and ( $\alpha$ -1,2)Fucosyltransferase Activity - Clone mH1-12 was selected with a monoclonal anti-H antibody that recognizes type II blood group H structures ( $\text{Fu}\alpha(1,2)\text{Gal}\beta(1,4)\text{GlcNAc-R}$ ). Binding of this antibody to mH1-12 cells is blocked when the antibody is preincubated with the type II H hapten 2'-fucosyllactose ( $\text{Fu}\alpha(1,2)\text{Gal}\beta(1,4)\text{Glc}$ ). By contrast, preincubation of the

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anti-H antibody with L-fucose, or with N-acetyllactosamine or lactose, at identical concentrations, does not inhibit binding of the antibody to mH1-12 cells. When a different monoclonal anti-H antibody (BE2) previously shown to be specific for type II H structures was used in these experiments, the inventor also observed inhibition of binding with 2'-fucosyllactose, but not with the other haptens. These studies indicate that mH1-12 cells express cell surface glycoconjugates with terminal  $\text{Fu}\alpha(1,2)\text{Gal}$  linkages.

Additional evidence for the presence of this linkage was obtained by using the linkage-specific blood group A ( $\alpha$ -1,3)GalNAc transferase purified from human plasma. This glycosyltransferase has an absolute requirement for blood group H acceptors containing fucosyl  $\alpha(1,2)\text{galactoside}$  as the terminal nonreducing group. It catalyzes the addition of N-acetylgalactosamine in  $\alpha$ -1,3 linkage to the galactose moiety of this structure to construct blood group A-reactive molecules of the form  $\text{GalNA}\alpha(1,3)[\text{Fu}\alpha(1,2)]\text{Gal}$ . Generation of blood group A-reactive determinants on the surface of mH1-12 cells by the action of blood group A glycosyltransferase would provide confirmation of the presence of terminal  $\text{Fu}\alpha(1,2)\text{Gal}$  linkages inferred by the results of the type II H hapten inhibition study.

Formalin-fixed mH1-12 cells were incubated with preparations of the blood group A ( $\alpha$ -1,3)GalNAc transferase and its nucleotide sugar substrate (UDPGalNAc, 1 mM,

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approximately 20-fold above  $K_m$  for UDP-GalNAc) in a buffer supporting activity of this enzyme. The cells were then probed for the presence of newly synthesized, surface-localized blood group A determinants by indirect immunofluorescence using a monoclonal anti-A antibody.

After a 4-h incubation with the A ( $\alpha$ -1,3)GalNAc transferase and its substrate, blood group A determinants were detectable on the surface of the cells. No staining with anti-A antibody was observed in control reactions done in the absence of either UDP-GalNAc or group A transferase. L cells showed no binding of anti-H or anti-A under any of these conditions.

The inventor also stained A enzyme-treated cells with anti-H antibody to test for loss of surface expressed H structures. These should be "masked" with N-acetylgalactosamine molecules that are attached by the A enzyme to the galactose of the  $Fuc\alpha(1,2)Gal$  linkage. After a 4-h incubation with both A enzyme and its substrate, the staining generated by the anti-H antibody was only slightly diminished. However, after the conversion reaction was allowed to proceed for 24 h, essentially complete elimination of cell surface H reactivity was seen. This is coincident with continued expression of strong A reactivity. Cells treated for 24 h with a control reaction mixture containing the A enzyme but without substrate exhibited strong anti-H staining. This indicates that loss of H reactivity seen after

the 24-h reaction was not due to destruction of H structures by glycohydrolase or protease activities contaminating the group A enzyme preparation. Loss of H reactivity after prolonged incubation thus represents "masking" of H structures by the A enzyme-catalyzed attachment of  $\alpha$ -1,3-linked N-acetylgalactosamine. These data indicate that mH1-12 cells express cell surface glycoconjugates terminating with authentic H  $\text{Fu}\alpha(1,2)\text{Gal}$  linkages.

Assays of extracts prepared from mH1-12 cells confirmed that these cells express  $\alpha$ -(1,2)fucosyltransferase activity.  $\alpha$ -Fucosidase digestion of the fucosylated product of these reactions confirmed the  $\alpha$  anomeric linkage of the attached fucose.

Analysis of the Human DNA Sequences in the Primary Transfectant - Southern blot analysis was used to determine if the mH1-12 cell line contains human DNA sequences. The BLUR8 Alu sequence was used to detect human sequences. With the hybridization and washing conditions used, the human Alu probe did not cross-hybridize with mouse sequences, but was able to detect the equivalent of a few copies of an Alu sequence that had been added to mouse L cell DNA (10  $\mu\text{g}$ ). By comparison, the A431 DNA sample (3 ng) displayed a diffuse yet relatively intense hybridization signal expected for the highly repetitive interspersed Alu sequences. Under these conditions, the inventor was able to detect significant amounts of human sequences in the genome of mH1-12 cells (500

ng). This analysis indicates that, as expected, the genome of mH1-12 cells contains roughly 1000 kb of human DNA.

**Isolation of Multiple Secondary Transfectants That Express Cell Surface Type II H Antigen and ( $\alpha$ -1,2)Fucosyltransferase Activity** - The inventor wished to be able to identify within the large number of human sequences in the genome of mH1-12 cells specific human sequences that mediate expression of its H-positive phenotype. To reduce the amount of extraneous human DNA, the inventor used DNA prepared from the mH1-12 cell line to generate "secondary" transfectant "libraries," and screened these libraries for transfectants that expressed the H structure. The inventor expected that H-expressing secondary transfectants so identified would each have a small number of human DNA restriction fragments identifiable within their genomes. The inventor sought to isolate several independent secondary transfectants since it was anticipated that human sequences linked to H-determining gene(s) should be a subset of these human fragments, identifiable as characteristic restriction fragments of identical sizes in each independently derived H-expressing secondary transfectant.

Genomic DNA prepared from mH1-12 cells was contransected with pSV2-neo into L cells. Four different secondary libraries were generated in this way (Table I).



Table I

Estimated frequencies of H antigen-positive  
transfectants in six independent libraries

Frequencies are expressed as one independent H-expressing transfectant isolated/number of plates of transfectants screened. For libraries screened by cell sorting or panning, this is a minimum estimate since these immunoselection procedures do not allow discrimination between H-expressing sibs and independently derived H-positive transfectants; at least one independent H-expressing transfectant was present in each of these libraries. For the primary library mH1, and secondary libraries mHs1, mHs3, mHs4, and mHs5, each plate contained approximately 2000 independent transfectants, as determined from transfection efficiency estimates (see "Experimental Procedures"). For the mHs2 secondary library, inspection of the plates prior to screening indicated that approximately 50 colonies were present on each plate. Clones s2-1 and s2-2 were isolated with the rosette procedure from two separate plates. Clone s2-3 was isolated by panning a population of cells representing approximately 650 independent transfectant colonies.

Library name	Source of transfected DNA	Added selection plasmid	Fraction of transfectants expressing H determinants
mH1 (primary)	A431 Cells	pSV2-neo	$\geq 1/30$ dishes
mHs1 (secondary)	mH1-12	pSV2-neo	$\geq 1/30$ dishes
mHs3 (secondary)	mH1-12	pSV2-neo	$\geq 1/10$ dishes
mHs4 (secondary)	mH1-12	pSV2-neo	$\geq 1/10$ dishes
mHs5 (secondary)	mH1-12	pSV2-neo	$\geq 1/10$ dishes
mHs2 (secondary)	mH1-12	None	$\geq 1/-650$ colonies
			$\geq 1/-50$ colonies (rosette s2-1)
			$\geq 1/-50$ colonies (rosette s2-2)

Each library was independently screened for H-expressing transfectants using the panning procedure. Prior to the third round of panning, FACS analysis indicated that each of these libraries contained H antigen-positive cells (1-60% of the cells bound anti-H antibody). Sequential selection by panning was continued until 50-90% of the cells exhibited the H--positive phenotype. Clonal cell lines (S1-11 and S3-6) were then established from populations derived from the mHs1 and mHs2 libraries; more than 95% of the cells in these lines exhibited bright staining with anti-H antibody. Libraries mHs4 and mHs5 were not subjected to the cell cloning procedure but were instead subjected to additional selections by panning. After a total of 11 rounds of selection, approximately 95% (selected from mHs4 library) and 50% (selected from mHs5 library) of these cells exhibited H antigen expression.

The calcium phosphate transfection procedure the inventor used for constructing the mH1-12 cell line occasionally results in physical linkage of selectable plasmid sequences with the transfected genomic DNA sequences that determine the desired phenotype. Linkage of pSV2-neo sequences to human DNA sequences determining the H-positive phenotype in the mH1-12 primary transfectant would simplify identification of H-expressing secondary transfectants and would facilitate isolation of the relevant transfected sequences by molecular cloning procedures. As a test of such linkage, the inventor generated a secondary library, mHs2, by transfecting L cells with DNA prepared from the mH1-12 primary transfectant. The transfection was done without the addition of exogenous pSV2-neo DNA. This procedure yielded approximately 50 independent G418 transfectants on each of fifteen 10-cm dishes. This represents a 40-fold reduction in the number of G418-resistant colonies obtained, relative to the numbers generated when secondary libraries were generated with the addition of pSV2-neo DNA (~2000 G418 resistant cells/dish).

This mHs2 library was initially screened with an in situ rosette procedure for rapid identification of transfectant colonies that bound anti-H antibody (see "Experimental Procedures" below). Culture dishes containing approximately 50 colonies each were screened with this method 16 days after transfection and prior to any other manipulations. A single rosette-positive colony was identified on two of the 15 dishes

tested. These two independent H-positive colonies were isolated with cloning rings and an H-expressing cell line (s2-1 and s2-2) was established from each. An additional, independent, clonal H-expressing transfectant (s2-3) was isolated by harvesting the colonies on the other 13 dishes and subjecting these cells to selection by panning and then cell cloning.

Cotransfection of unlinked single-copy markers occurs at a frequency less than 1%. The frequencies of coexpression of G418 resistance and the H phenotype the inventor observed in the mHs2 library (Table I) are consistent with the possibility that the two markers are linked in the primary transfectant. Alternatively, these frequencies could be explained by cotransfection of unlinked markers present in multiple copies in the primary transfectant. In any event, the frequencies of H-expressing transfectants observed in the primary and secondary libraries (Table I) indicate that the H-positive phenotype expressed by these transfectants is determined by a single transfected locus.

The anti-H reactive surface molecules on a representative H-expressing secondary transfectant (clone s2-2) were shown to be authentic H  $\text{Fu}\alpha(1,2)\text{Gal}$  linkages, using analyses identical to those used for the H antigen-positive primary transfectant mH1-12. Extracts prepared from s2-2 cells were found to contain  $(\alpha-1,2)\text{fucosyltransferase}$  activity.  $(\alpha-1,2)\text{Fucosyltransferase}$  activity was also found in extracts

prepared from each of the other H-expressing secondary transfectants.

**Independent H-Expressing Secondary Transfectants Have Common Restriction Fragments Containing Human DNA Sequences -**

The inventor anticipated that the genome of each H-positive secondary transfectant would contain a relatively small amount of human DNA and that this DNA would include human sequences controlling expression of the ( $\alpha$ -1,2)fucosyltransferase found in each. In principle, one or more characteristic restriction fragment(s) generated by these sequences should be identifiable in every transfectant. Conversely, irrelevant human sequences should exhibit a random restriction pattern in the secondary transfectants. The inventor therefore isolated genomic DNA from each transfectant, digested these DNAs with various restriction enzymes, and subjected these digests to Southern blot analysis. Restriction fragments containing human DNA sequences were detected with the BLUR8 Alu probe. A number of DNA restriction fragments are present in each clonal secondary transfectant; the aggregate amount of human genomic DNA present in these cells is estimated to be between 25 and 55 kb. The genome of each clonal secondary transfectant contains a characteristic pair of human DNA EcoRI restriction fragments with sizes of 2.7 and 3.4 kb. These fragments are also evident in pools of cells selected by panning from libraries mHs4 and mHs5. Similar analyses indicate that the genome of each H-expressing secondary transfectant contains

common 1.5 and 1.9 kb PstI fragments and a common 2.8-kb PvuII human DNA restriction fragment. These observations imply that DNA sequences within or linked to these characteristic human restriction fragments are associated with expression of the cell surface H Fuc $\alpha$ (1,2)Gal linkages used to select these transfectants, and are thus implicated in the expression of the ( $\alpha$ -1,2)fucosyltransferase found in these cells.

To further confirm that the common human DNA sequences in the transfectants direct ( $\alpha$ 1,2)fucosyltransferase expression in these cells, molecular cloning procedures were used to isolate these fragments and then test their function in a mammalian transient expression system. The two human DNA EcoRI fragments previously found to be associated with expression of the H phenotype in the H-expressing secondary transfectants were isolated from mini genomic libraries prepared in a lambda phage vector, using the secondary transfectant s2-2 (see "Experimental Procedures"). To determine if these fragments contained sufficient genetic information to direct synthesis of ( $\alpha$ 1,2)fucosyltransferase, these were first individually subcloned into the mammalian expression cosmid vector pWE15. This vector contains the SV40 origin of replication, enabling it to replicate efficiently as an episome in COS-1 cells. The resulting plasmids contained either the 3.4 kb EcoRI (plasmid pH3.4) or the 2.7 kb EcoRI fragment (plasmid pH2.7). These plasmids were then individually introduced into COS-1 cells by DEAE-dextran

transfection (see "Experimental Procedures") and the transfected cells were subsequently assayed for ( $\alpha$ 1,2)fucosyltransferase activity. The inventor found no detectable ( $\alpha$ 1,2)fucosyltransferase activity in mock transfected COS-1 cells or in COS-1 cells transfected with pH2.7. However, COS-1 cells transfected with plasmid pH3.4 expressed significant amounts of ( $\alpha$ 1,2)fucosyltransferase activity.  $\alpha$ -Fucosidase digestion of the fucosylated phenyl- $\beta$ -D galactoside product generated by this extract confirmed the alpha anomeric configuration of the attached fucose (see "Experimental Procedures").

The pH-activity profile of this ( $\alpha$ 1,2)fucosyltransferase mirrors the profiles determined for the ( $\alpha$ 1,2)fucosyltransferases found in fractionated human serum, in A431 cells, and in the H-expressing mouse transfectants. Likewise, the apparent Michaelis constants exhibited by the recombinant enzyme expressed in COS-1 cells (GDP-fucose  $K_m$ =17.5  $\mu$ M; phenyl- $\beta$ -D-galactoside  $K_m$ =4.4 mM) are essentially the same as those determined by the inventor for the ( $\alpha$ 1,2)fucosyltransferases in fractionated human serum, and in each of the cell lines he analyzed. Considered together, these results are consistent with the proposal that human DNA sequences within the 3.4 kb *Eco*RI fragment encode an ( $\alpha$ 1,2)fucosyltransferase, and that these sequences encompass part or all of the human blood group H ( $\alpha$ 1,2)fucosyltransferase gene. The 3.4 kb *Eco*RI fragment in

pH3.4, the 2.7 kb *EcoRI* fragment in pH2.7, and DNA sequence adjacent to the 3.4 kb fragment, were sequenced to provide SEQ ID NO:5.

To characterize the nature of these cloned human genomic DNA sequences, the inventor first isolated various restriction fragments from the insert in plasmid pH3.4 and tested these for their ability to identify transcripts in the H-expressing, stable transfectants, and in a human cell line (A431) that also expresses H determinants and a cognate ( $\alpha$ 1,2)fucosyltransferase. He found that a 1.2 kb *HinfI* restriction fragment from the insert in pH3.4 identifies a single, relatively non-abundant 3.6 kb transcript in A431 cells. This probe also detects transcripts in the H-expressing mouse L cell transfectants, but not in the non-transfected parental L cells.

A cloned cDNA that directs expression of cell surface H structures and an ( $\alpha$ 1,2)fucosyltransferase. The inventor used the 1.2 kb *HinfI* fragment and colony hybridization to isolate two hybridization positive cDNA clones from an A431 cell cDNA library. To test the cloned cDNAs for their ability to determine expression of surface-localized H antigen and a cognate ( $\alpha$ 1,2)fucosyltransferase activity, a plasmid was constructed (pCDM7- $\alpha$ (1,2)FT, see "Experimental Procedures") that consisted of the largest cDNA insert cloned into the mammalian expression vector pCDM7, in the sense orientation with respect to the vector's enhancer-promoter sequences.



Flow cytometry analysis of COS-1 cells transfected with PCDM7- $\alpha(1,2)$ FT indicates that this cDNA determines expression of cell surface H molecules. Moreover, COS-1 cells transfected with pCDM7- $\alpha(1,2)$ FT, but not cells transfected with pCDM7, express substantial quantities of an  $(\alpha 1,2)$ fucosyltransferase activity. The inventor determined the apparent Michaelis constant exhibited by this  $(\alpha 1,2)$ fucosyltransferase for an artificial acceptor (phenyl- $\beta$ -D-galactoside) that is specific for this enzyme and that can discriminate between the human H and Se  $(\alpha 1,2)$ fucosyltransferases. This apparent  $K_m$  (2.4mM) is nearly identical to the apparent  $K_m$  the inventor (3.1 mM,) and others (4.6 mM, 6.4 mM, 1.4 mM) have determined for the blood group H  $(\alpha 1,2)$ fucosyltransferase. Moreover, this apparent  $K_m$  is also very similar to the one exhibited by the  $(\alpha 1,2)$ fucosyltransferase in extracts prepared from COS-1 cells transfected with pH3.4 (4.4 mM). This apparent  $K_m$  is distinct from the one exhibited by an  $(\alpha 1,2)$ fucosyltransferase found in human milk enzyme (15.1 mM), that is thought to represent the  $(\alpha 1,2)$ fucosyltransferase encoded by the Se locus. These data demonstrate that the cDNA in plasmid pCDM7- $\alpha(1,2)$ FT determines expression of an  $(\alpha 1,2)$ fucosyltransferase whose kinetic properties reflect those exhibited by the human H blood group  $(\alpha 1,2)$ fucosyltransferase.

The cDNA sequence predicts a Type II transmembrane glycoprotein. The cDNA insert in pCDM7- $\alpha(1,2)$ FT is 3373 bases pairs long. Its corresponding transcript is 3.6 kb in length,

suggesting that this cDNA is virtually full-length. Two potential initiator codons are found within its first 175 nucleotides. Only the second of these, however, is embedded within a sequence context associated with mammalian translation initiation. The methionine codon initiates a long open reading frame that predicts a protein of 365 amino acids (SEQ ID NO:6), with a calculated Mr of 41,249 Da. This open reading frame is colinear with the open reading frame found in the 3.4 kb *EcoRI* fragment in pH3.4. Hydropathy analysis of the predicted protein sequence indicates that it is a Type II transmembrane protein, as noted for several other cloned glycosyltransferases. This topology predicts an 8 residue NH<sub>2</sub>-terminal cytosolic domain, a 17 residue hydrophobic transmembrane domain flanked by basic amino acids, and a 340 amino acid COOH-terminal domain that is presumably Golgi-resident and catalytically functional. Two potential N-glycosylation sites are found in this latter domain suggesting that this sequence, like other glycosyltransferases, may exist as a glycoprotein. No significant similarities were found between this sequence and other sequences in protein or DNA databases (Protein Identification Resource, Release 21.0, and Genbank, Release 60.0), with the exception of a 642 bp sequence within the 3'-untranslated segment of the cDNA that is similar to the human Alu consensus sequence. Moreover, the inventor identified no significant sequence similarities

between this cDNA sequence or its predicted protein sequence, and those of other cloned glycosyltransferase cDNAs.

The protein encoded by the cDNA is an ( $\alpha$ 1,2)fucosyltransferase. The results of the expression experiments present above, when considered together with the domain structure predicted by the cDNA sequence, are consistent with the presumption that it encodes an ( $\alpha$ 1,2)fucosyltransferase. Nonetheless, the inventor wished to directly confirm this, and thus exclude the possibility that it instead encodes a molecule that trans-determines this enzyme activity. The inventor therefore fused the putative catalytic domain of the predicted protein to a secreted form of the IgG-binding domain of *Staph. aureus* protein A (see Experimental Procedures) in the mammalian expression vector pPROTA, to yield the vector pPROTA- $\alpha$ (1,2)FT<sub>c</sub>. By analogy to similar constructs the inventor has prepared with other cloned glycosyltransferases (detailed *infra*), he expected that if the cDNA sequence actually encodes an ( $\alpha$ 1,2)fucosyltransferase, then plasmid pPROTA- $\alpha$ (1,2)FT<sub>c</sub> would generate a secreted, soluble, and affinity purifiable ( $\alpha$ 1,2)fucosyltransferase. Indeed, conditioned media prepared from a plate of COS-1 cells transfected with pPROTA- $\alpha$ (1,2)FT<sub>c</sub> contained a total of 5,790 units of ( $\alpha$ 1,2)fucosyltransferase activity, whereas a total of 1,485 units were found to be cell-associated. Moreover, virtually 100% of the released ( $\alpha$ 1,2)fucosyltransferase activity was specifically retained by IgG-Sepharose, and most

could be recovered after exhaustive washing of this matrix. By contrast, the inventor found that most of the activity in COS-1 cells transfected with pCDM7- $\alpha$ (1,2)FT was cell-associated (3450 units), with only trace amounts of activity in the conditioned media prepared from these cells (~80 units). Virtually none of this latter activity bound to either matrix. Extracts prepared from COS-1 cells transfected with vector pCDM7 or vector pPROTA did not contain any detectable cell-associated or released ( $\alpha$ 1,2)fucosyltransferase activity. These data demonstrate that the cDNA insert in pCDM7- $\alpha$ (1,2)FT encodes an ( $\alpha$ 1,2)fucosyltransferase, and that information sufficient to generate a catalytically active ( $\alpha$ 1,2)fucosyltransferase is encompassed with the 333 amino acids distal to the putative transmembrane segment.

Experimental Procedures for Example I. "Cloning and Expression of a DNA Sequence Encoding ( $\alpha$ 1,2)Fucosyltransferase".

The term "L cells" used throughout the text refers to the mouse Laprt<sup>tk</sup> cell line.

Lactose, N-acetyllactosamine, 2'-fucosyllactose (Fuca(1,2) Gal $\beta$ (1,4)Glc), UDP-GalNAc, phenyl- $\beta$ -D-galactoside, and Ficoll 400 were obtained from Sigma. L-Fucose was from Pfanstiehl Labs (Waukegan, IL). UDP[1-<sup>3</sup>H]N-acetylgalactosamine (8.7 Ci/mmol) and D-[U-<sup>14</sup>C]mannose (239 mCi/mmol) were from

DuPont-New England Nuclear. D-[2-<sup>3</sup>H]mannose (16.3 Ci/mmol), L-[6-<sup>3</sup>H]fucose (72 Ci/mmol), L-[1-<sup>14</sup>C]fucose (58.7 mCi/mmol), GDP[U-<sup>14</sup>C]- $\beta$ -L-fucose (268 mCi/mmol), and [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) were from Amersham Corp. Nonradioactive GDP-fucose was kindly provided by Dr. Eric Holmes (Seattle). FITC-ECA was obtained from E-Y Labs (San Mateo, CA). Plasmid pSV2-neo was obtained from Dr. David Chaplin (Washington University, St. Louis). Restriction enzymes (New England Biolabs or Boehringer Mannheim) were used according to the manufacturer's instructions.

#### Antisera:

Monoclonal anti-H, anti-A, and anti-B antibodies (mouse IgM) were purchased from Chembiomed, Ltd. (Alberta, Canada). Monoclonal anti-H antibody BE2 was prepared from BE2 hybridoma cell culture supernatants (see below). Anti-mouse IgM and FITC-labeled antimouse IgM (both antigen-affinity purified, goat) were from Sigma.

#### Cell Lines and Culture:

Mouse Laprt<sup>tk</sup> cells were obtained from Dr. David Chaplin. Human A431 cells were from Dr. Brian Whiteley and Dr. Luis Glaser (Washington University, St. Louis). BE2 cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (Hyclone, Logan, UT).

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Transfected cells were grown in media containing G418 (GIBCO) at 400  $\mu\text{g/ml}$  (active drug).

Preparation of Genomic DNA:

High molecular weight genomic DNA was prepared from cultured cells by standard methods. Samples of genomic DNA were electrophoresed through 0.3% agarose gels buffered in Tris-acetate-EDTA to confirm their integrity and to estimate the average size of the molecules in the preparations.

Transfections:

The calcium phosphate precipitation method was used to transfect mouse L cells with human genomic DNA. Cells ( $5 \times 10^5$ /100-mm dish) were incubated overnight with DNA precipitates (20-30  $\mu\text{g}$  of genomic DNA and 1  $\mu\text{g}$  of pSV2-neo). No exogenous pSV2-neo DNA was included in transfections that generated the mHs2 secondary library. The cells were fed fresh media the following day and were placed under G418 selection the next day. Transfection efficiencies were estimated by harvesting transfected cells 1 day after addition of DNA and plating duplicate serial dilutions of the cell suspensions. One set of dilutions was grown under G418 selection, and the other was grown in the absence of antibiotic to allow the derived transfection efficiencies to be corrected for plating efficiency. After 2 weeks of growth, colonies were counted after staining with 0.2% methylene blue in 50% methanol.

Approximately 2000 independent transfectants were typically obtained by transfecting  $5 \times 10^5$  cells on a 100-mm dish.

Immunologic Selection of H-expressing Transfectants:

Transfectants were removed from culture dishes by incubating them with PBS containing 3 mM EDTA. Detached cells were washed and resuspended in staining medium (10 mM HEPES, pH 7.4, 0.1% sodium azide, 2% fetal calf serum in Dulbecco's modified Eagle's medium). The cells were kept at 4°C throughout the panning or cell sorting procedures. Cell cloning was done by plating cells at low density, allowing individual cells to form colonies, and isolating individual colonies with cloning cylinders.

Panning - Bacteriological culture dishes (Falcon 1007, 60 mm) were prepared for panning. Goat antimouse IgM was coupled to the dishes by incubating them overnight at 4°C with 4 ml of antibody solution diluted to 10 µg/ml in 50 mM Tris, pH 9.5. The antibody solution was aspirated, and the dishes were washed twice with PBS. The dishes were then blocked by incubating them at room temperature for at least 1 h with PBS containing 1 mg/ml bovine serum albumin. Dishes were then used immediately or were stored indefinitely at 4°C. The dishes were washed three times with PBS prior to use.

Cells to be panned were resuspended at a concentration of  $10^7$ /ml, in staining media containing anti-H antibody at 10 µg/ml. The cells were incubated for 30 min at 4°C, and unbound

antibody was removed by pelleting the cells through 10 ml of PBS containing 1 mM EDTA, 0.1% sodium azide, and 2% Ficoll 400. After centrifugation, the supernatant was carefully aspirated, and the cells were resuspended at  $10^6$ /ml, in staining media. Three-ml aliquots of this cell suspension were applied to 60-mm panning dishes coated with goat anti-mouse IgM. The dishes were then incubated for 1 h at 4°C, and were then rinsed 5 times with serum-free Dulbecco's modified Eagle's medium to remove nonadherent cells. Fresh, serum-replete media was added to the dishes, and they were returned to the tissue culture incubator. The next day, adherent cells were removed with trypsin-EDTA and were replated on standard tissue culture dishes. These cells were grown for 10-18 days prior to the subsequent selection.

Cell Sorting - Transfectants were prepared for FACS analysis by incubating them for 30 min at 4°C with monoclonal IgM anti-H antibody (10  $\mu$ g/ml in staining media) or with a control monoclonal IgM anti-B antibody (10  $\mu$ g/ml) in staining media). The cells were then washed in ice-cold staining media, and incubated for 30 min at 4°C in staining media containing fluorescein-conjugated goat antimouse IgM at 40  $\mu$ g/ml. The cells were washed, resuspended in staining media, and subjected to analysis by the FACS (Coulter Electronics model Epics C). Samples were gated on forward and 90° light scatter to eliminate dead cells from analysis. H-expressing cells were collected aseptically into staining media and then



returned to culture for 10-18 days before additional selections.

**Rosette Procedure** - A rosetting method was used to identify colonies of transfectants that bound anti-H antibody. This was done on 100-mm dishes containing isolated colonies comprised of approximately 100-300 cells. Plates of colonies were first rinsed twice with PBS and were then incubated for 1 h at 4°C with 4 ml of mouse IgM monoclonal anti-H antibody at 10 µg/ml in PBS, 2% fetal calf serum, 0.1% sodium azide. The plates were then rinsed three times with PBS, and were incubated for 30 min at 4°C with 4 ml of human erythrocytes conjugated with goat anti-mouse IgM. (Goat anti-mouse IgM was coupled to human blood group O red cells with chromic chloride. After conjugation, the red cells were washed with PBS, diluted to a 0.2% v/v suspension in PBS, 2% fetal calf serum, 0.1% sodium azide, and were used immediately.) Afterwards, the suspension of erythrocytes was carefully aspirated, and the plates were gently rinsed with PBS and examined on a light box. Colonies that had bound anti-H antibody were macroscopically visible as "rosettes" consisting of red cells adherent to the colonies.

**Purification of Blood Group A ( $\alpha$ -1,3)GalNAc Transferase:**

Group A transferase was isolated from human blood group A plasma by affinity chromatography on Sepharose 4B (Sigma lot no. 104F0334). Column fractions containing the peak of enzyme

activity were pooled, mouse serum albumin (Behring Diagnostics >98%) was added to a 1% concentration, and aliquots were stored at -80°C until use. The activity of the final preparation was determined by a standard radiochemical method. One enzyme unit is defined as one nmol of GalNAc transferred to 2'-fucosyllactose acceptor/h.

#### Paper Chromatography:

Descending paper chromatography was performed using Whatman No. 3mm or Whatmann No. 1 in the following solvent systems: Solvent A, ethyl acetate/pyridine/water (10:4:3); Solvent B pyridine/water/ethyl acetate (10:11:5:36), upper phase. The dried chromatograms were cut into 1-cm strips and radiolabeled compounds were eluted with water. Radioactivity in an aliquot of each eluate was determined by scintillation counting.

#### Analysis of L Cell GDP-Fucose Content:

To identify GDP-fucose in mouse L cells, cells ( $2.5 \times 10^6$ ) were labeled for 3 days with 250  $\mu$ Ci of D-[2- $^3$ H]mannose in 30 ml of complete media. Cells were harvested and extracted with 60% ethanol for 5 min in a boiling water bath. The aqueous extract containing nucleotide sugars was concentrated under vacuum and resuspended in a small volume of water. Unlabeled GDP-mannose (270 nmol) and GDP-fucose (130 nmol) were added as internal standards, as the mixture was subjected to gel

filtration chromatography on a Sephadex G-25 column (0.9 X 42-cm) equilibrated in 50 mM ammonium acetate. The eluate was monitored at 268 nm; fractions containing GDP-fucose and GDP-mannose were pooled, concentrated under vacuum, and resuspended in 200  $\mu$ l of water. This was subjected to fractionation by HPLC on a weak anion exchange column (AX300, 4 mm X 24 cm, Pierce Chemical Co.). The sample was applied to the HPLC column equilibrated in 100 mM triethylamine acetate, pH 7.0, and was eluted with a linear gradient from 100 to 300 mM triethylamine acetate, pH 7.0 in 50 min at a flow rate of 2 ml/min. The eluant was monitored at 268 nm, and fractions (0.5 ml) were collected for scintillation counting and subsequent analysis. The unlabeled nucleotide sugar internal standards were identified by their characteristic elution times (GDP-fucose, 35.5 min. approximately 800 cpms; GDP-mannose, 33.0 min. approximately 1000 cpms). The radioactive peaks corresponding to the fractions coeluting with unlabeled GDP-fucose and GDP-mannose were subjected to hydrolysis with 0.1 N HCl for 45 min at 100°C. These were then fractionated by descending paper chromatography on Whatman No. 3MM in solvent B for 20 h, in parallel with L-[<sup>14</sup>C]fucose and D-[<sup>14</sup>C]mannose standards. In each case, approximately 30% of the counts hydrolyzed were recovered as the appropriate monosaccharide.

Analysis of Fucose-labeled Glycopeptides:

Radiolabeled glycopeptides were prepared and analyzed. Mouse L cells ( $2 \times 10^6$ ) were labeled for 3 days with 200  $\mu$ Ci of L-[6- $^3$ H]fucose in 20 ml of complete media. Cells were harvested and extracted with chloroform, and then water, and the pellet remaining after the final extraction was subjected to exhaustive digestion with Pronase (Behring Diagnostics). This was then desalted by gel filtration chromatography on Sephadex G-25-80. This material was concentrated under vacuum, and an aliquot was hydrolyzed in 0.1 N HCl at 100°C for 45 min. The hydrolysate was then subjected to descending paper chromatography on Whatman No. 3MM in solvent B for 20 h, in parallel with an L-[ $^{14}$ C]fucose standard. Approximately 26% of the counts present in the macromolecular material were released and cochromatographed with the radiolabelled fucose standard.

Assay of GDP-L-Fucose: $\beta$ -D-Galactoside 2- $\alpha$ -L-Fucosyltransferase:

Cultured cells were washed with PBS, pelleted by centrifugation, and resuspended in a small volume of 25 mM sodium phosphate, pH 6.1, containing 0.5% Triton X-100. Volumes were adjusted to achieve a protein concentration of approximately 5 mg/ml (BCA method, Pierce Chemical Co.). Extracts were typically assayed immediately after preparation. The standard assay contained 5-20  $\mu$ l of enzyme solution

(typically 30-100  $\mu$ g of cell extract protein or 15  $\mu$ l of serum) in 40  $\mu$ l of 25 mM potassium phosphate, 0.1% Triton X-100, 3  $\mu$ M GDP-[ $^{14}$ C]fucose, 25 mM phenyl- $\beta$ -D-galactoside, and 5 mM ATP. The pH of the reaction mixture was adjusted to a final measured pH of 6.1. Assays were terminated after an appropriate period of time by the addition of 20  $\mu$ l of ethanol. The mixture was then centrifuged at 15,000 X g for 5 min. The supernatant was collected, spotted on Whatman No. 1, and subjected to fractionation by descending paper chromatography for 4 h in Solvent A. Radioactivity was then determined as described above. In all cases, parallel reactions were done in the absence of added phenyl- $\beta$ -D-galactoside acceptor to allow correction for endogenous acceptor molecules. No products of endogenous acceptor molecules cochromatographing with fucosylated phenyl- $\beta$ -D-galactoside were identified in any samples.

$\alpha$ -Fucosidase Digestion:

[ $^{14}$ C]Fucosylphenyl- $\beta$ -B-galactoside (approximately 10,000 cpm) was isolated from fucosyltransferase assays by paper chromatography, concentrated from the water eluate under vacuum, and resuspended in 5  $\mu$ l of water. This was digested with 0.025 units of bovine kidney  $\alpha$ -L-fucosidase (EC 3.2.1.51) in a final volume of 20  $\mu$ l containing 5 mM sodium citrate, pH 6.0, at 37°C for 1 h. This mixture was then fractionated by descending paper chromatography on Whatman No. 1 in Solvent A

for 4 h. Products of the digestions were identified by comparison to parallel separations of authentic L-[<sup>14</sup>C]fucose, and purified [<sup>14</sup>C]fucosylphenyl- $\beta$ -D-galactoside synthesized by human plasma H ( $\alpha$ -1,2)fucosyltransferase.

Indirect Immunofluorescence:

Immunofluorescence was performed on cells plated on 8-well tissue culture chamber slides (Lab-Tek). Cells were plated at a density of  $5 \times 10^4$ /well 24 h prior to analysis. Anti-H and anti-A primary antibodies were diluted in PBS containing bovine serum albumin at 2 mg/ml, to a final concentration of 10  $\mu$ g/ml. Cell-bound primary antibodies were detected with FITC-conjugated goat anti-mouse IgM, diluted to 40  $\mu$ g/ml in PBS containing 2 mg/ml bovine serum albumin.

Hapten Inhibition - Plated cells were washed twice with PBS, and were incubated for 30 min at 4°C with 100  $\mu$ l of diluted anti-H antibody, or with 100  $\mu$ l of diluted antibody containing different oligosaccharide haptens, each at a concentration of 20 mM. The chambers were then washed twice, and 100  $\mu$ l of FITC-conjugated goat anti-mouse IgM was added. After 30 min at 4°C, the chambers were washed three times with PBS, and the cells were fixed at room temperature for 10 min in 3.7% formaldehyde in PBS. The cells were washed twice with PBS, the chambers were removed, and the slide was mounted in PBS containing 25% glycerol. Cells were examined by

fluorescence microscopy using a Zeiss Axiophot photomicroscope equipped with fluorescence epiillumination.

**Labeling of Intact Cells with Human Blood Group A ( $\alpha$ -1,3)GalNac Transferase** - Cells plated in culture slide chambers were washed twice with 150 mM NaCl and were then fixed for 10 min at room temperature in 3.7% formaldehyde in 150 mM NaCl. The cells were washed three times with 150 mM NaCl and were then incubated with 100  $\mu$ l of complete transferase reaction mixture or a control mixture. The complete transferase mixture consisted of 150 mM NaCl, 15 mM  $MnCl_2$ , 50 mM sodium cacodylate, pH 6.8, 0.2% bovine serum albumin, 1 mM UDP-GalNac, and 1.14 units of human blood group A ( $\alpha$ -1,3)GalNac transferase. Control mixtures consisted of identical components except for the omission of either UDP-GalNac or blood group A ( $\alpha$ -1,3)GalNac transferase. Incubations were performed at 37°C and were terminated after either 4 or 24 h by washing the chambers twice with PBS. The cells were then analyzed by indirect immunofluorescence using mouse monoclonal anti-A or anti-H antibodies, as described above for the hapten inhibition studies.

#### Southern Blotting:

Genomic DNA was digested to completion with restriction enzymes (8 units/ $\mu$ g DNA, overnight digestion). The restriction fragments were fractionated by electrophoresis through 0.6% agarose gels buffered in Tris-borate-EDTA. The

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DNA fragments were then transferred to nylon membranes (Hybond-N, Amersham Corp.), according to the standard Southern blotting method. Blots were prehybridized at 39°C for at least 2 h in 50% formamide, 5 SSC (1 X SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0, 1 X PE is 50 mM Tris, pH 7.5, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate, 0.2% polyvinylpyrrolidone ( $M_r$  40,000), 0.2% Ficoll ( $M_r$  400,000), and 5 mM EDTA), and 150  $\mu$ g/ml denatured salmon sperm DNA.

Hybridizations were done in the same solution at 39°C for at least 16 h. Blots were washed four times at room temperature in 2 X SSC, 0.1% sodium dodecyl sulfate, and then once for 30 min at 65°C in 0.75 X SSC, 0.5% sodium dodecyl sulfate. The BLUR8 probe consisted of a 300-base pair BamHI segment isolated from the BLUR8 plasmid. This fragment was subjected to two cycles of gel purification prior to labeling to ensure that it was free from contaminating plasmid sequences. Probes were labeled with [ $\alpha^{32}$ P]dCTP to a specific activity of at least  $5 \times 10^8$  cpm/ $\mu$ g using the random priming method.

Paper Chromatography      Descending paper chromatography was performed using Whatman No. 40 in ethyl acetate/pyridine/water (10:4:3; Solvent A) or using Whatman No. 3MM in 95% ethanol/1 M ammonium acetate (7:3; Solvent B).  $^{14}$ C-Labelled compounds were located by autoradiography of dried chromatograms. Alternatively, the dried chromatograms were cut into 1 cm strips and the radiolabelled compounds were eluted with water.



An aliquot of each eluate was mixed with scintillation cocktail and radioactivity was determined in a scintillation counter.

Preparation of Radiolabelled Standards [ $^{14}\text{C}$ ]Fucose-1-phosphate was prepared by enzymatic cleavage of GDP-[ $^{14}\text{C}$ ]fucose (1 nmol) with snake venom phosphodiesterase (EC 3.1.4.1, 1  $\mu\text{l}$ , 0.003 units, Boehringer-Mannheim) in 20  $\mu\text{l}$  of 100 mM Tris-HCl, pH 8 at 37°C for 1 h. The reaction was then fractionated by descending paper chromatography on Whatman No. 3MM using Solvent B for 20 h in parallel with GDP-[ $^{14}\text{C}$ ]fucose and [ $^{14}\text{C}$ ]fucose. [ $^{14}\text{C}$ ]Fucose-1-phosphate ( $R_{\text{fucose}} = 0.45$ ) was then eluted from the chromatogram with water and concentrated under vacuum. [ $^{14}\text{C}$ ]Fucosylphenyl- $\beta$ -D-galactoside was generated from GDP-[ $^{14}\text{C}$ ]fucose (3  $\mu\text{M}$ ) and phenyl- $\beta$ -D-galactoside (25 mM) by the action of ( $\alpha$ 1,2)fucosyltransferase activity in human serum, using the reaction conditions described below for assay of  $\alpha$ (1,2)fucosyltransferase. The products of this reaction were fractionated by descending paper chromatography on Whatman No. 40 for 4 h in Solvent A.

[ $^{14}\text{C}$ ]Fucosylphenyl- $\beta$ -D-galactoside was identified by autoradiography of the dried chromatogram and was then eluted from the paper with water and concentrated under vacuum. Alternatively, [ $^{14}\text{C}$ ]fucosylphenyl- $\beta$ -D-galactoside was isolated from fucosyltransferase assay mixtures using the Sep-Pak procedure described below.

Cell Lines and Cell Culture COS-1 cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Protein Determinations Protein concentrations were determined by the BCA method (Pierce Chemical Co.) according to the manufacturer's instructions. Bovine serum albumin was used as the standard.

Preparation of Cell Extracts Cells were washed with PBS, removed from culture dishes with a rubber policeman, and pelleted by centrifugation. Cell pellets were resuspended in 2 volumes of cold 1% Triton X-100 (Surfactants X-100, Pierce Chemical Co.) and sonicated for 15 seconds using a Branson sonicator equipped with a micro tip at 50% power. These extracts were either assayed immediately, or were stored at -20°C until use. Under these conditions, enzyme activity in the frozen extracts was stable for several weeks, but deteriorated rapidly on repeated freezethawing. Tissues from C3H mice were isolated, minced with a razor blade, suspended in 2 volumes of 1% Triton X-100 and sonicated as described above, centrifuged at 1500 x g for 5 min and the supernatants were collected. Mouse intestinal mucosa extracts were prepared by everting the small intestine onto a thin polypropylene rod, scraping the mucosa cells into phosphate

buffered saline, and collecting the cells by centrifugation at 1500 x g. Extracts were then prepared as described above.

#### Partial Purification of Mouse Intestinal

##### ( $\alpha$ 1,2)Fucosyltransferase

Preliminary experiments indicated that extracts prepared from mouse intestinal mucosa contained large amounts of an activity that hydrolyzed GDP-fucose in an acceptor-independent manner. Since substrate hydrolysis interfered with accurate determination of ( $\alpha$ 1,2)fucosyltransferase activity, intestinal extracts were fractionated by anion exchange chromatography to separate GDP-fucose hydrolysis activity from ( $\alpha$ 1,2)fucosyltransferase activity. All procedures were performed at 40°C. Two ml of the Triton-solubilized extract was made 10 mM in Tris-HCl, pH 7.6, and treated for 5 min with 2 ml (bed volume) of DEAE-cellulose (DE52, Whatman) previously equilibrated with 10 mM Tris-HCl, pH 7.6. The enzyme solution was filtered, made 10 mM in sodium phosphate buffer and the pH was adjusted to 6.1. Approximately 47% of the enzyme activity present in the initial extracts was recovered after the DEAE-cellulose fractionation procedure. A second treatment with DEAE-cellulose resulted in substantial loss of enzyme activity with no significant reduction in GDP-fucose hydrolysis activity (data not shown). When used under standard fucosyltransferase assay conditions, but in the

absence of added acceptor, this preparation hydrolyzed less than 2% of the GDP-fucose initially present in the reaction.

Ammonium Sulfate Fractionation of Serum Serum was prepared from freshly drawn blood obtained from a non-Secretor individual. The blood was clotted in a glass tube at 37°C for 1 h and was immediately fractionated by ammonium sulfate precipitation exactly as described. The 20%-40% ammonium sulfate fraction was dialyzed against 2 changes of 4 liters of water (8 h each) at 4°C. Assay of ( $\alpha$ 1,2)fucosyltransferase was done immediately. Alternatively, the fractionated serum was aliquoted and stored at -20°C until use.

#### Ion Exchange Chromatography of Human Milk

##### ( $\alpha$ 1,2)Fucosyltransferase

( $\alpha$ 1,2)Fucosyltransferase was isolated from human milk by published procedures. Briefly, 300 ml of milk from a Se-positive donor was defatted by centrifugation and was extensively dialyzed against 20 mM sodium cacodylate, pH 6.0. This was applied to a column (2.6 x 115 cm) of sulfopropyl-Sephadex equilibrated in 20 mM cacodylate, pH 6.0. The column was then washed with 1 liter of 20 mM cacodylate, pH 6.0, and was eluted with a linear gradient made from 1.5 liter each of 20 mM cacodylate, pH 6.0, and 500 mM NaCl in 20 mM cacodylate, pH 6.0. Fractions (13 ml) were collected and assayed for ( $\alpha$ 1,2)fucosyltransferase activity using

phenyl- $\beta$ -D-galactoside acceptor as described below. Fractions 130-144 contained ( $\alpha$ 1,2)fucosyltransferase activity. These were combined, concentrated to 1 ml by ultrafiltration in an Amicon stirred cell fitted with a YM5 membrane (MW cutoff = 5,000), and were then equilibrated against cold deionized water. The concentrated enzyme was aliquoted and stored at  $-80^{\circ}\text{C}$ .

Synthesis and Characterization of GDP- $\beta$ -L-fucose and GDP- $\alpha$ -L-fucose

GDP- $\beta$ -L-fucose was synthesized and purified by modifications of the method of Nunez et al. (Nunez et al, Can. J. Chem., 59, 2086-2095, 1981). The modified method described here eliminates the need to separate the anomeric 1-fucopyranosyl phosphates by differential crystallization prior to subsequent synthetic steps. Separation of anomeric products is effected by HPLC subsequent to the last synthetic procedure.

Pyridine and tetrahydrofuran (Aldrich) used in the synthesis were boiled under reflux over calcium hydride, distilled and stored over 4 Å molecular sieves. All evaporations were performed under reduced pressure on a rotary evaporator, with a bath temperature below  $35^{\circ}\text{C}$ . L-Fucose was acetylated exactly as described in Nunez et al (Can. J. Chem., 59, 2086-2095, 1981). The resulting mixture of 2,3,4-tri-O-acetyl- $\alpha$ - and  $\beta$ -L-fucopyranoses (2.7 g, 9.27 mmol)

was phosphorylated using *o*-phenylene phosphochloridate. The crude reaction product containing a mixture of anomeric 1-fucopyranosyl phosphates was fractionated on a Dowex-1 column ( $\text{HCO}_3^-$ , 20-50 mesh, 1.5 x 23 cm) preequilibrated in water. After application of the anomeric mixture, the column was washed with water (500 ml) and eluted with 400 mM triethylammonium bicarbonate buffer, pH 7.5 (250 ml). The triethylammonium bicarbonate eluant was evaporated to a thick syrup under reduced pressure. The syrup was dissolved in water and evaporated to dryness. This partially purified anomeric mixture of 1-fucopyranosyl phosphates (bis-triethylammonium salt, crude yield 70%) was used for the synthesis of GDP- $\beta$ -L-fucose. The anomeric 1-fucopyranosyl phosphates (200 mg) were first repeatedly dissolved in dry pyridine and then evaporated to dryness in vacuo. Guanosine 5'-phosphomorpholidate (400 mg) was then added to the dried anomeric 1-fucopyranosyl phosphates. This mixture was subjected to repeated resuspension in dry pyridine and evaporation to dryness in vacuo. The reaction mixture was then suspended in dry pyridine (15 ml) and was incubated at room temperature. The formation of GDP- $\beta$ -L-fucose was monitored daily by high performance liquid chromatography (HPLC) on a weak anion exchange column (AX 300, 4.6 mm x 22 cm, Pierce Chemical Co.). An aliquot (10  $\mu$ l) from the reaction mixture was evaporated to dryness, dissolved in water and mixed with GDP- $^{14}\text{C}$ fucose (2000 cpm). The sample was then

applied to the HPLC column equilibrated in water, and was eluted with a linear gradient from 100% water to 250 mM triethylammonium acetate, pH 7.0, in 60 min at a flow rate of 2 ml/min. The eluant was monitored at 268 nm, and 0.5 ml fractions were collected for scintillation counting to identify the co-injected GDP-[<sup>14</sup>C]fucose. Three peaks absorbing at 268 nm eluted at approximately 180 mM triethylammonium acetate. The retention time of the first peak (38.5 min) was identical to the retention time of GDP- $\alpha$ -L-fucose (see below). The second peak eluted at 40.6 min, and coeluted with the GDP- $\beta$ -L-[<sup>14</sup>C]fucose standard. A third small peak eluting at 42.4 min was not identified. The reaction was judged to be essentially complete after 5 days. The proportion of  $\alpha$  to  $\beta$  anomer in the final reaction mixture was found to be approximately 2:3. The reaction was then evaporated to dryness and dissolved in water. GDP- $\beta$ -L-fucose was purified from this solution on a preparative Hydropore AX column (21.4 mm x 25 cm, Rainin Instruments Co.). Aliquots of the aqueous solution were applied to the column equilibrated in water, and the sample was eluted with a linear gradient of 100% water to 200 mM triethylammonium acetate, pH 7.0, in 45 min, at a flow rate of 10 ml/min. The eluant was monitored at 268 nm. GDP- $\alpha$ -L-fucose eluted at 36.2 min. GDP- $\beta$ -L-fucose eluted at 38.5 min. The GDP- $\beta$ -L-fucose peak was collected and evaporated to dryness under reduced pressure. Ammonium acetate was removed by repeated co-evaporation with water.

The compound that co-eluted with GDP-[ $^{14}\text{C}$ ]fucose, and that we tentatively identified as GDP- $\beta$ -L-fucose, was subjected to negative ion fast atom bombardment mass spectrum analysis (xenon), using a VG mass spectrometer (model 7B-250S). The results ( $m/z$   $[\text{M-H}]^-$  588) were consistent with this identification. The compound was then analyzed by proton decoupled  $^{13}\text{C}$  NMR spectroscopy to confirm the anomeric configuration of the fucose, and to further establish the identity of the compound as GDP- $\beta$ -L-fucose. Proton decoupled  $^{13}\text{C}$  NMR spectra were obtained on a Bruker WM 360, operating at 909.5 MHz and a sweep width of 221 ppm with 32K data points. Probe temperature was  $38 \pm 1^\circ\text{C}$ . Resonances are reported in ppm relative to tetramethylsilane. This analysis yielded the following spectral data:  $^{13}\text{C}$  NMR (40  $\mu\text{M}$  in  $\text{D}_2\text{O}$ , pH 7.0); guanine:  $\delta$ 140.27 (C1 and C8), 156.46 (C2), 154.38 (C4), 118.85 (C5), 161.46 (C6); ribosyl moiety: 89.36 (C1), 76.06 (C2), 73.6 (C3), 86.4 (C4), 67.8 (C5); fucosyl moiety: 100.9 (C1), 75.09 (C2), 73.94 (C3), 73.71 (C4), 73.02 (C5), 17.35 (C6). The distinction between anomeric forms of GDP-L-fucose is based upon the chemical shift of anomeric C(1) of the fucose ring. The C(1) resonance of the fucose ring in the  $\beta$ -anomer is shifted downfield (100.9 ppm) relative to the resonance of the anomeric C(1) in the  $\alpha$  anomer (98.31 ppm, see below). The values obtained for this compound are essentially identical to those reported by Nunez et al. for GDP- $\beta$ -L-fucose. The resonances attributable to the C atoms of



the guanine, ribose and fucose ring are also in agreement with the literature values.

GDP- $\alpha$ -L-fucose was synthesized in a manner similar to that described for the preparation of GDP- $\beta$ -L-fucose, except that the dicyclohexylammonium salt of ( $\alpha$ -L-(-)fucose-1-phosphate (Sigma) was used instead of the anomeric mixture of 1-fucopyranosyl phosphates. Analysis and purification of GDP- $\alpha$ -L-fucose was performed by HPLC using the same conditions described for GDP- $\beta$ -L-fucose. The purified compound, tentatively identified as GDP- $\alpha$ -L-fucose, was subjected to analysis by negative ion fast atom bombardment mass spectroscopy. The results ( $m/z$   $[M-H]^-$  588) were consistent with this assignment. The subsequent analysis of the compound by  $^{13}\text{C}$  NMR spectroscopy yielded the following spectral data:  $^{13}\text{C}$  NMR (50  $\mu\text{M}$  in  $\text{D}_2\text{O}$ , pH 6.97); guanine: 6140.27 (C1 & C8), 156.46 (C2), 154.41 (C4), 118.84 (C5), 161.46 (C6); ribosyl moiety: 89.35 (C1), 76.04 (C2), 71.98 (C3), 86.4 (C4), 67.8 (C5); fucosyl moiety: 98.31 (C1), 70.36 (C2), 72.99 (C3), 74.3 (C4), 70.36 (C5), 17.88 (C6). The C(1) resonance of the fucose ring in this compound (98.31 ppm) is consistent with an  $\alpha$  anomeric configuration, as reported by Nunez et al. The resonances attributable to the C atoms of the guanine and the ribose ring are also in agreement with the literature values reported for these atoms in GDP- $\beta$ -L-fucose. GDP- $\beta$ -L-fucose was found to be inactive as a substrate for ( $\alpha$ 1,2)fucosyltransferase.

Assay of GDP-L-fucose: $\beta$ -D-galactoside:2- $\alpha$ -L-fucosyltransferase

Fucosyltransferase assays were performed by a modification of the procedure reported by Chester et al. (Chester et al, Eur. J. Biochem., 69: 583-593, 1976). The standard assay contained GDP-[ $^{14}$ C]fucose (3  $\mu$ M), phenyl- $\beta$ -D-galactoside (25 mM), ATP (5 mM) and the enzyme solution (1-10  $\mu$ l) in 20  $\mu$ l of 25 mM sodium phosphate buffer, pH 6.1. Based upon preliminary assays, amounts of added enzyme activities were adjusted to ensure that reactions were linear throughout the period of incubation (4 h for fractionated serum, 2 h for each of the other enzyme preparations). Under these conditions, less than 15% of the substrate was consumed during the incubation period. For the determination of pH optima, assays were buffered with 25 mM sodium acetate, sodium phosphate, or Tris-HCl, using concentrated solutions of these buffers previously adjusted to various pH values. The final pH value of each reaction was determined with a micro pH probe. In assays to determine the apparent  $K_m$  values for GDP-fucose, GDP-[ $^{14}$ C]fucose was diluted with unlabeled GDP-fucose to a final specific activity of 26.3 mCi/mmol. The concentration of GDP-fucose in this stock solution was calculated from the UV absorbance at 254 nm of an aliquot diluted in water. The molar extinction coefficient of GDP ( $\epsilon$ =13800 at 254 nm, pH 7.0) was used for this calculation since the extinction coefficient of GDP-fucose is not known. This stock was then used to yield variable GDP-fucose

concentrations (3 - 300  $\mu$ M) in the assays. The concentration of phenyl- $\beta$ -D-galactoside in these assays was 25 mM, for all but the milk-derived enzyme. This was assayed in the presence of 75 mM phenyl- $\beta$ -D-galactoside since the apparent  $K_m$  exhibited by this enzyme for this acceptor is 15.1 mM. In assays to determine apparent  $K_m$  values for phenyl- $\beta$ -D-galactoside, the concentration of the acceptor was varied from 0.5 to 100 mM. GDP-[ $^{14}$ C]fucose was present in these assays at a concentration of 3  $\mu$ M. The relatively low specific activity of commercially available GDP-[ $^{14}$ C]fucose, and its cost, necessitated the use of a substrate concentration well below its  $K_m$  in these experiments. All assays were performed at 37°C. Assays were terminated after an appropriate period of time by addition of 20  $\mu$ l of ethanol, followed by dilution with 1 ml of water. The mixture was then centrifuged at 15,000 x g for 5 min. The supernatant contained virtually 100% of the radiolabelled product; this was collected, and an aliquot was used for separation and quantitation of the fucosylated product using the hydrophobic interaction chromatography method described below. All assays were performed in duplicate or triplicate and included parallel incubations done in the absence of acceptor. The values obtained in the absence of exogenous acceptor were subtracted to correct for the presence of endogenous acceptor molecules. This background acceptor "activity" was always less than 3% of the radioactivity incorporated into the

phenyl- $\beta$ -D-galactoside acceptor. Apparent Michaelis constants were derived from Lineweaver-Burke plots of acceptor concentration-rate determinations. Intercepts were calculated by the least squares estimation method.

A separation procedure based upon hydrophobic interaction chromatography was developed for rapid processing of large numbers of samples. This procedure effects separation of [ $^{14}$ C]fucosylphenyl- $\beta$ -D-galactoside product from GDP-[ $^{14}$ C]fucose on disposable C-18 Sep-Pak cartridges (Waters-Millipore). The bottom of the Sep-Pak cartridge is mounted in one hole of a two-holed rubber stopper, and a 5 ml syringe is attached to the top of the cartridge. The other hole in the stopper is attached to a vacuum source (approximately 350 mm of Hg). The mounted Sep-Pak cartridge is prepared by washing with 5 ml of acetonitrile, followed by 5 ml of water. This is done by separately pipetting each wash solution into the attached syringe barrel and drawing it through the cartridge under the vacuum created when the rubber stopper (with attached vacuum line) is pressed onto the top of a 20 ml plastic scintillation vial. The contents of the syringe are aspirated through the cartridge into the vial. The fucosyltransferase reaction sample is then loaded into the syringe and aspirated through the cartridge into a fresh scintillation vial. The [ $^{14}$ C]fucosylphenyl- $\beta$ -D-galactoside is retained on the Sep-Pak cartridge. The cartridge is then washed sequentially by aspiration with three 2 ml portions of water, collecting each

wash into a new scintillation vial. The [ $^{14}\text{C}$ ]fucosylphenyl- $\beta$ -D-galactoside product is then eluted by aspiration with three 2 ml portions of 50% acetonitrile. Scintillation cocktail (10 ml, Biosafe II, Research Products International) is added to each vial to obtain a clear solution and the radioactivity in each is determined by scintillation counting. Radioactivity eluting in fractions 1-4 represents GDP-[ $^{14}\text{C}$ ]fucose, fucose-1-phosphate, and [ $^{14}\text{C}$ ]fucose present either as a contaminant in the substrate as obtained from the manufacturer (approximately 1%), or that is formed by substrate or product hydrolysis. Fractions 5-7 represent [ $^{14}\text{C}$ ]fucosylphenyl- $\beta$ -D-galactoside. The recovery of radioactivity with this procedure exceeds 97%. Reconstruction experiments showed that recovery of pure [ $^{14}\text{C}$ ]fucosylphenyl- $\beta$ -D-galactoside is essentially 100%. Separation and recovery are independent of pH and enzyme source, and are tolerant of detergents (Triton X-100 and Lubrol-PX) at concentrations up to 2%. Product separation by this method requires approximately one minute per sample. Sep-Pak cartridges have been reused indefinitely with no deterioration in performance.

The Sep-Pak method does not separate GDP-[ $^{14}\text{C}$ ]fucose from [ $^{14}\text{C}$ ]fucose-1-phosphate and thus cannot detect nucleotide pyrophosphatase activity that may consume GDP-fucose while generating fucose-1-phosphate and/or fucose. ATP (5 mM) was therefore included in all assays to inhibit nucleotide

pyrophosphatase activity. Effective inhibition of pyrophosphatase activity was confirmed by descending paper chromatography analysis of mock fucosyltransferase assays using each enzyme preparation. Each enzyme source was incubated for 1 to 4 h at 37°C in 20  $\mu$ l of standard assay cocktail, but in the absence of phenyl- $\beta$ -D-galactoside. After the reactions were terminated, an aliquot of each was spotted on Whatman No. 3MM, fractionated by descending paper chromatography for 20 h in solvent B, and radioactivity was determined as described above. Authentic GDP-[ $^{14}$ C]fucose, [ $^{14}$ C]fucose-1-phosphate, and L-[ $^{14}$ C]fucose standards were chromatographed in parallel. This system separates GDP-fucose, fucose-1-phosphate, and fucose. It therefore allows quantitation of the amount of GDP-[ $^{14}$ C]fucose remaining at the end of the incubation, and thus provides an estimate of pyrophosphatase activity. Under the standard assay conditions, more than 97% of the GDP-[ $^{14}$ C]fucose initially present in each mock reaction remained unhydrolyzed and available for transglycosylation.

Each enzyme preparation was also tested for  $\alpha$ -fucosidase activity that could hydrolyze the [ $^{14}$ C]fucosylphenyl- $\beta$ -D-galactoside product and prevent accurate determination of ( $\alpha$ 1,2)fucosyltransferase activity. Enzyme preparations were incubated with purified [ $^{14}$ C]fucosylphenyl- $\beta$ -D-galactoside (7000 cpm, 10 pmol) in fucosyltransferase assay buffer, but in the absence of added GDP-[ $^{14}$ C]fucose or phenyl-

$\beta$ -D-galactoside. These reactions were incubated at 37°C for various times, and were then fractionated by the Sep-Pak method to determine the amount of remaining [ $^{14}$ C]fucosylphenyl- $\beta$ -D-galactoside. Fractionated human serum and mouse intestinal mucosa extracts each contained significant  $\alpha$ -fucosidase activity. However, inclusion of 10 mM L-fucose in the reaction cocktail effectively inhibited fucosidase activity in these enzyme preparations. Therefore 10 mM L-fucose was included when assaying these enzyme sources. Significant amounts of  $\alpha$ -fucosidase activity were not detected in any of the other cell extracts; product hydrolysis never exceeded 1%/h under the standard assay conditions. Reconstruction experiments with these extracts showed that L-fucose at a concentration of 10 mM does not alter the activity of ( $\alpha$ 1,2)fucosyltransferase.

In aggregate, these experiments showed that, under the conditions used to assay fractionated human serum, human milk, and mouse intestinal mucosa, or crude cell extracts, the quantity of [ $^{14}$ C]fucosylphenyl- $\beta$ -D-galactoside product determined reflected true enzymatic activity, because both product hydrolysis and acceptor-independent hydrolysis of the GDP-fucose substrate were negligible.

Digestion of [ $^{14}$ C]Fucosylphenyl- $\beta$ -D-galactoside with  
 $\alpha$ -Fucosidase

[<sup>14</sup>C]Fucosylphenyl- $\beta$ -D-galactoside (approximately 10,000 cpm) was digested with 0.025 units of bovine kidney  $\alpha$ -L-fucosidase (EC 3.2.1.51, Sigma) in a final volume of 20  $\mu$ l containing 5 mM sodium citrate pH 6.0, at 37°C for 1 h. This mixture was then fractionated by descending paper chromatography on Whatman No. 40 in Solvent A for 4 h. The products of the digestion were identified by comparison to parallel separations of L-[<sup>14</sup>C]fucose and [<sup>14</sup>C]fucosylphenyl- $\beta$ -D-galactoside standards.

Isolation of Human DNA Restriction Fragments from Transfectant Clone s2-2

High molecular weight genomic DNA was isolated from the H-expressing secondary transfectant s2-2, digested to completion with *Eco*RI, and fractionated through a 1% agarose gel buffered in tris-borate-EDTA. The region of the gel containing the 2.7 kb and the 3.4 kb human *Eco*RI fragments was divided into 3 mm slices and the DNA in these was isolated by electroelution. Aliquots of the size-fractionated DNA were analyzed by Southern blotting with a radiolabelled Alu probe (BLUR8), using hybridization and wash conditions described above. Fractions containing either the 2.7 kb or the 3.4 kb fragment were used separately to prepare phage libraries in lambda gt11. These libraries were screened with a radiolabelled BLUR8 probe. Positive phages isolated from a tertiary screen were used to prepared phage DNA, and phages



containing either the 2.7 kb *EcoRI* fragment or the 3.4 kb *EcoRI* fragment were identified by Southern blotting. The 3.4 kb or the 2.7 kb inserts were released from the phage arms by *EcoRI* digestion, purified by agarose gel electrophoresis and electroelution, and individually subcloned between the *EcoRI* sites in pWE15.

COS-1 Cell Transfection Plasmid DNAs were transfected into COS-1 cells by the DEAE-dextran procedure. Seventy two hours after transfection, cells were harvested, extracts were prepared as described above, and extracts were subjected to assays for  $\alpha(1,2)$ fucosyltransferase activity, for GDP-fucose hydrolysis activity, and for  $\alpha$ -fucosidase activity.

Isolation of Human  $\alpha(1,2)$ FT cDNA Clones.  $1.8 \times 10^6$  recombinant clones from an A431 cell cDNA mammalian expression library were screened by colony hybridization, using a  $^{32}\text{P}$ -labeled 1.2 kb *HinfI* fragment of pH3.4 as a probe. Filters were hybridized for 18 hours at 42°C in a hybridization solution described above, washed, and subjected to autoradiography. Two hybridization-positive colonies were obtained and isolated via two additional rounds of hybridization and colony purification. Preliminary sequence analysis of the inserts in both hybridization-positive cDNA clones indicated that they each were in the anti-sense orientation with respect to the pCDM7 expression vector promoter sequences. The largest

insert was therefore re-cloned into pCDM7 in the sense orientation for expression studies, and the resulting plasmid was designated pCDM7- $\alpha$ (1,2)FT.

Flow Cytometry Analysis. COS-1 cells were transfected with plasmid DNAs using the DEAE-dextran procedure described above. Transfected cells were harvested after a 72 hour expression period and stained either with mouse IgM anti-H monoclonal antibody (Chembiomed; 10  $\mu$ g/ml) or with a mouse IgM anti-Lewisa monoclonal antibody (Chembiomed; 10  $\mu$ g/ml). Cells were then stained with fluorescein-conjugated goat anti-mouse IgM antibody (Sigma; 40  $\mu$ g/ml) and subjected to analysis by flow cytometry.

Northern and Southern Blotting A431 poly(A)-plus RNA (10  $\mu$ g/lane) was subjected to Northern blot analysis. Genomic DNA (10  $\mu$ g/lane) was subjected to Southern blot analysis. Blots were probed with  $^{32}$ P-labeled 1.2 kb *Hinf*I fragment of pH3.4.

DNA Sequence Analysis. The insert in pCDM7- $\alpha$ (1,2)FT was sequenced by the method of Sanger using T7 DNA polymerase (Pharmacia) and 20-mer oligonucleotide primers synthesized according to the sequence of the cDNA insert. Sequence analyses and data base searches were performed using the Microgenie Package (Beckman) and the Sequence Analysis

Software Package of the University of Wisconsin Genetics  
Computer Group.

Assay of  $\alpha(1,2)$ fucosyltransferase Activity. Cell extracts, conditioned medium from transfected COS-1 cells, and IgG-Sepharose-bound enzyme were prepared and assayed for  $\alpha(1,2)$ fucosyltransferase activity by the methods described above. One unit of  $\alpha(1,2)$ fucosyltransferase activity is defined as 1 pmol product formed per hour. The apparent Michaelis constant for the acceptor phenyl- $\beta$ -D-galactoside was determined exactly as described above.

Construction and Analysis of a Protein A- $\alpha(1,2)$ FT Fusion

Vector. A 3196 bp *StuI/XhoI* segment of the cDNA insert containing the putative catalytic domain and 3'-untranslated sequences was isolated from pCDM7- $\alpha(1,2)$ FT. This fragment was blunt-ended using Klenow enzyme and ligated to phosphorylated and annealed oligonucleotides (CGGAATTCCCCACATGGCCTAGG, <sup>(SEQ ID NO 5)</sup> CCTAGGCCATGTGGGGAATTCCG) <sup>(SEQ ID NO 6)</sup> designed to reconstruct the coding sequence between the putative transmembrane segment proximal to the *StuI* site, corresponding to amino acids 33 through 365 of SEQ ID NO:6. The ligated fragment was gel purified, digested with *EcoRI* and then gel purified again. This *EcoRI* linkered fragment was ligated into the unique *EcoRI* site of pPROTA. One plasmid, designated pPROTA- $\alpha(1,2)$ FT<sub>c</sub>, containing a single insert in the correct orientation, was analyzed by DNA

sequencing to confirm the sequence across the vector, linker and insert junctions. Plasmids pPROTA- $\alpha(1,2)$ FTC, pPROTA, pCDM7- $\alpha(1,2)$ FT, or pCDM7 were transfected into COS-1 cells. Following a 72 hour expression period,  $\alpha(1,2)$ FT activities in the media, associated with cells, bound to a Sepharose IgG matrix, or to a control Sepharose matrix, were quantitated.

Example II.     Cloning and expression of a DNA Sequence  
                  Encoding a UDP-Gal: $\beta$ -D-Gal(1,4)-D-GlcNAc  
                   $\alpha(1,3)$ galactosyltransferase (DNA SEQ ID NO:3,  
                  Protein SEQ ID NO:4)

A Gene Transfer Approach to Isolate Cloned, Functional,  $\beta$ -D-galactosyl-1,4-N-acetyl-D-glucosaminide  $\alpha$ -1,3-galactosyltransferase cDNAs. Tissue- and cell-specific expression of surface-localized terminal Gal( $\alpha$ 1-3)Gal linkages is associated with expression of cognate ( $\alpha$ 1-3)GTs that catalyze a transglycosylation reaction between UDP-Gal and N-acetyllactosamine. COS-1 cells construct surface-expressed polylactosamine molecules that can function as an acceptor substrate for ( $\alpha$ 1-3)GT but do not express this enzyme or its surface-localized product (see below). The inventor therefore expected that cloned cDNAs encoding an ( $\alpha$ 1-3)GT would, if expressed in COS-1 cells, generate the surface-localized oligosaccharide product of that enzyme [terminal Gal( $\alpha$ 1-3)Gal linkages]. Moreover, these particular transfectants could be isolated by virtue of adherence to plates coated with a lectin

(GS I-B<sub>4</sub>) that specifically binds terminal Gal( $\alpha$ 1-3)Gal linkages. A standard transient expression system was used for this approach since it provides for the rescue of transfected cDNAs that determine the expression of cell surface molecules on COS-1 cells and allows the facile construction of large cDNA libraries in a mammalian expression vector.

Isolation of a Cloned cDNA That Determines Expression of GS I-B<sub>4</sub> Binding Activity in Transfected COS-1 Cells. Mouse F9 teratocarcinoma cells express an ( $\alpha$ 1-3)GT, and this enzyme activity increases concomitant with retinoic acid-induced differentiation of these cells. The inventor therefore prepared a cDNA expression library from retinoic acid-differentiated F9 cells and screened this library for cDNAs that determine expression of GS I-B<sub>4</sub> binding activity in transfected COS-1 cells. One plasmid (pCDM7- $\alpha$ GT) was isolated that, when transfected into COS-1 cells, determined expression of surface molecules that directed specific adherence of cells to culture dishes coated with GS I-B<sub>4</sub>. Fluorescence-activated cell sorting analysis confirmed these observations. COS-1 cells transfected with pCDM7- $\alpha$ GT, but not cells transfected with pCDM7, stained brightly with fluorescein isothiocyanate-conjugated GS I-B<sub>4</sub>. This staining could be inhibited with raffinose, a hapten for this lectin. These observations indicate that pCDM7- $\alpha$ GT determines de novo expression of surface-localized molecules recognized by GS I-B<sub>4</sub> and thus

expression of terminal Gal( $\alpha$ 1-3)Gal linkages on cell surface oligosaccharides.

cDNA Sequence Analysis Predicts a Protein with a Transmembrane Topology. The cDNA insert in pCDM7- $\alpha$ GT (SEQ ID NO:3), Fig. 2, is 1500 base pairs long and contains a single long open reading frame in the sense orientation with respect to pCDM7 promoter sequences. Three methionine codons are found within the first 15 codons of this reading frame; the inventor assigned the most proximal of these as the initiator codon, based on Kozak's rules for mammalian translation initiation. This reading frame predicts a protein of 394 amino acids in length (SEQ ID NO:4), Fig. 2, with a molecular mass of 46,472 Da. Hydropathy analysis indicates that this protein has features of a type II transmembrane molecule that is topologically identical to that predicted for two other mammalian glycosyltransferases. This topology predicts a 41-amino-acid, cytoplasmically oriented,  $\text{NH}_2$ -terminal segment; a single transmembrane domain consisting of a 19-amino-acid hydrophobic segment flanked by basic residues; and a large (presumably catalytic) COOH-terminal domain that would ultimately be targeted to the lumen of the Golgi. Two potential N-glycosylation sites are present, indicating that this protein, like other glycosyltransferases, may be synthesized as a glycoprotein. This cDNA sequence contains a long 5' untranslated region, with ATG codons at -90 and -251, suggesting that translational control mechanisms may

participate in the regulation of expression of this sequence. This is reminiscent of another mammalian glycosyltransferase whose transcript also contains upstream ATG codons. The putative NH<sub>2</sub>-terminal end of this protein lacks a characteristic cleavable signal sequence that may exist in one form of a murine  $\beta$ -1,4-galactosyltransferase.

Searches of the currently available protein and nucleic acid data bases (Protein Identification Resource, Release 21.0 and GenBank, Release 60.0) identified no sequences with significant similarity to the ( $\alpha$ 1-3)GT DNA sequence, including the sequences of a murine  $\beta$ -1,4-galactosyltransferase and a rat  $\alpha$ -2,6-sialyltransferase.

**Expression of a Catalytically-Active, Secreted Protein A-( $\alpha$ 1-3)GT Fusion Protein.** The inventor wished to confirm that this cDNA encodes an ( $\alpha$ 1-3)GT and to simultaneously exclude the formal possibility that it instead encodes a trans-acting molecule that induces ( $\alpha$ 1-3)GT activity by interaction with an endogenous gene, transcript, or protein. Therefore, sequences corresponding to the putative catalytic domain of this protein (residues 63-394 of SEQ ID NO:4) were fused in-frame to a secretable form of the IgG binding domain of Staphylococcus aureus protein A in the mammalian expression vector pPROTA yielding the vector pPROTA- $\alpha$ GT<sub>c</sub>. This vector was then tested for its ability to express a catalytically active, secreted and soluble protein A-( $\alpha$ 1-3)GT fusion protein.

COS-1 cells transfected with the pCDM7 vector or with the pPROTA vector generated no detectable cell-associated or released ( $\alpha$ 1-3)GT activity. By contrast, extracts prepared from COS-1 cells transfected with pCDM7- $\alpha$ GT or with the pPROTA- $\alpha$ GT<sub>c</sub> vector contained 4574 and 20,500 total units, respectively, of ( $\alpha$ 1-3)GT activity. Moreover, conditioned media prepared from cells transfected with pCDM7- $\alpha$ GT or pPROTA- $\alpha$ GT<sub>c</sub> contained soluble ( $\alpha$ 1-3)GT activity amounting to 4,155 units or 50,438 units, respectively. Importantly, the released activity generated by pPROTA- $\alpha$ GT<sub>c</sub> could be specifically bound to a IgG-Sepharose matrix, whereas the released activity generated by pCDM7- $\alpha$ GT did not interact with this affinity adsorbent. These results indicate that this cloned cDNA encodes an ( $\alpha$ 1-3)GT, show that information sufficient to generate a catalytically active ( $\alpha$ 1-3)GT resides within the 332 amino acids distal to the putative transmembrane segment, and show that the catalytic domain can be affinity purified in an enzymatically active state as a portion of a bipartite fusion protein.

**Determination of the Structure of the Trisaccharide Product of the ( $\alpha$ 1-3)GT.** Exoglycosidase digestion was used to confirm the  $\alpha$ -anomeric linkage predicted for the oligosaccharide product generated by the recombinant enzyme. Radiolabeled trisaccharide product was prepared from UDP-[<sup>14</sup>C]Gal and N-acetyllactosamine by using the IgG-Sepharose-bound enzyme activity generated by pPROTA- $\alpha$ GT<sub>c</sub>.



Digestion of the HPLC-purified trisaccharide product with a galactosidase resulted in quantitative release of [ $^{14}\text{C}$ ]Gal, whereas the trisaccharide was completely resistant to  $\beta$ -galactosidase digestion.

To confirm that carbon 3 of the galactose in the N-acetyllactosamine acceptor is involved in the glycosidic linkage formed by the recombinant enzyme, the inventor prepared a [ $^3\text{H}$ ]Gal-labeled N-acetyllactosamine acceptor and incubated it with IgG-Sepharose-bound protein A-( $\alpha$ 1-3)GT activity and 1 mM UDP-Gal under the standard ( $\alpha$ 1-3)GT reaction conditions. The trisaccharide product of this reaction was purified and subjected to methylation analysis. Radioactive 2,4,6-trimethylgalactose was identified. Together, these results indicate that the recombinant enzyme can utilize UDP-Gal and N-acetyllactosamine as substrates to construct a trisaccharide product with the structure Gal( $\alpha$ 1-3)Gal( $\beta$ 1-4)-GlcNAc.

Northern Blot Analysis. The ( $\alpha$ 1-3)GT cDNA hybridizes to a single 3.6-kilobase transcript in F9 teratocarcinoma cells. The inventor's DNA sequence analysis of another cloned ( $\alpha$ 1-3)GT cDNA isolated by colony hybridization indicates that the insert in pCDM7- $\alpha$ GT represents the 5' end of this transcript. The remaining 2.1 kilobases of this transcript consist of 3' untranslated sequence not rescued by the expression cloning procedure.

The specific activity of ( $\alpha$ 1-3)GT in retinoic acid-differentiated F9 teratocarcinoma cells is approximately 4-fold higher than that in untreated F9 cells. Northern blot analysis indicates that steady-state levels of the ( $\alpha$ 1-3)GT transcript also increase concomitant with retinoic acid-induced differentiation of F9 teratocarcinoma cells. These results are similar to those reported in F9 cells with  $\beta$ -1,4-galactosyltransferase and suggest that the dynamic changes in cell surface oligosaccharide structures known to accompany in vitro differentiation of this cell line are associated with significant changes in glycosyltransferase gene expression.

Experimental Procedures for Example II. "Cloning and expression of a DNA sequence encoding a UDP-Gal: $\beta$ -D-Gal(1,4)-D-GlcNAc  $\alpha$ (1,3)galactosyltransferase".

Construction of an F9 Cell cDNA Library. A cDNA library was prepared from poly(A)<sup>+</sup> RNA isolated from retinoic acid-differentiated mouse F9 teratocarcinoma cells by using the procedure of Seed and Arruffo, and the mammalian expression vector pCDM7. pCDM7 is a progenitor of the vector pCDM8; pCDM7 lacks the polyoma sequences present in pCDM8, but is otherwise virtually identical. The library contained  $3 \times 10^6$  independent recombinants.

Isolation of a Mouse ( $\alpha$ 1-3)GT cDNA Clone. Plasmid DNA was prepared from an amplified portion of the library and was transfected in to COS-1 cells by using the DEAE-dextran

procedure. Forty samples of  $5 \times 10^5$  COS-1 cells (in 100-mm dishes) were transfected with 50  $\mu\text{g}$  of plasmid DNA each. After a 72-hr expression period, the transfected COS-1 cell monolayers were harvested and panned on dishes coated with Griffonia simplicifolia isolectin I B<sub>4</sub> (GS I-B<sub>4</sub>). Lectin panning dishes were prepared by using 10  $\mu\text{g}$  of GS I-B<sub>4</sub> per ml in phosphate-buffered saline (PBS) containing 0.1 mM Ca<sup>2+</sup> and 0.1 mM Mn<sup>2+</sup>. Plasmid DNA molecules were rescued from adherent cells and were transformed into the Escherichia coli host MC1061/P3. Plasmid DNA was prepared from these transformants and was subjected to an additional screening by the same procedure. Sib selection was subsequently used to screen for plasmids that determined expression of GS I-B<sub>4</sub> binding activity in COS-1 cells. E. coli transformants containing plasmid molecules rescued from the second screening were plated to yield 16 pools containing between 100 and 5000 colonies each. Plasmid DNAs were prepared from replica plates and were transfected separately into COS-1 cells, and the transfectants were screened by panning on GS I-B<sub>4</sub>-coated dishes. These experiments indicated that approximately 1 out of 1000 colonies contained cloned cDNAs determining the GS I-B<sub>4</sub>-binding phenotype. One "active" ~ 1000-colony pool was subdivided into several smaller pools, and these were each tested for GS I-B<sub>4</sub>-binding activity. Three subsequent rounds of sib selection with sequentially smaller, active pools identified a

single plasmid (pCDM7- $\alpha$ GT) that directed expression of GS I-B<sub>4</sub>-binding activity in COS-1 cells.

Flow Cytometry. COS-1 cells transfected with plasmid DNAs were harvested 48-72-hr after transfection. These were stained with either fluorescein isothiocyanate-conjugated GS I-B<sub>4</sub> at 10  $\mu$ g per ml in staining media or with fluorescein isothiocyanate-conjugated GS I-B<sub>4</sub> that had been previously incubated with 50 mM raffinose. Cells were then subjected to analysis by fluorescence-activated cell sorting as described above.

Northern Blotting and DNA Sequence Analysis. Northern blots were hybridized with radiolabeled pCDM7- $\alpha$ GT cDNA insert at 42°C in hybridization solution. DNA sequencing was performed with the chain termination method by using oligodeoxynucleotides synthesized according to the sequence within the cDNA insert. Sequence data base searches and analyses were performed with the Sequence Analysis Software Package published by the University of Wisconsin Genetics Computer Group.

Assay of ( $\alpha$ 1-3)GT and Product Characterization. Extracts were prepared from transfected COS-1 cells. Cell extracts, conditioned medium from transfected cells, or IgG-Sepharose-bound enzyme was assayed for ( $\alpha$ 1-3)GT. One unit of ( $\alpha$ 1-3)GT activity is defined as 1 pmol of Gal transferred to N-acetyllactosamine acceptor per hour.

HPLC-purified, radiolabeled oligosaccharide reaction products were subjected to digestion with either  $\alpha$ -galactosidase (Sigma, 20 mU) or  $\beta$ -galactosidase (Sigma, 1 mU) for 1 hr at 37°C in buffers recommended by the manufacturer. Reaction products were then fractionated by HPLC. Methylation analysis of reaction product(s) was carried out according to standard procedures.

Construction and Analysis of the Protein A-( $\alpha$ 1-3)GT Fusion Vector. A 1050-base-pair segment of the ( $\alpha$ 1-3)GT cDNA containing the putative catalytic domain was excised from pCDM7- $\alpha$ GT by digestion with EcoRI. This was cloned into the EcoRI site of pPROTA by using a double-stranded linker (5'-ACGGAATTCCGT-3') to maintain the correct reading frame, yielding plasmid pPROTA- $\alpha$ GT<sub>c</sub>.

Plasmids pPROTA- $\alpha$ GT<sub>c</sub>, pCDM7- $\alpha$ GT, and pPROTA were separately transfected into COS-1 cells. After a 72-hr expression period, the media were harvested and subjected to low-speed (300 x g for 8 min) and high-speed (100,000 x g for 1 hr) centrifugations. Supernatants were then adjusted to 0.05% Tween 20 and were incubated batchwise with 100  $\mu$ l of preequilibrated IgG-Sepharose or Sepharose 6B overnight at 4°C. The matrices were then thoroughly washed and used directly in ( $\alpha$ 1-3)GT assays.

Example III. Isolation of a cloned human cDNA that encodes a  
GDP-Fuc: $\beta$ -D-Gal (1,4/1,3)-D-GlcNac(/Glc)-  
 $\alpha$ (1,3/1,4)-fucosyltransferase. (DNA SEQ ID  
NO:1. Protein SEQ ID NO:2)

In one embodiment, the present invention provides a gene transfer system that allows isolating cloned cDNAs encoding functional  $\alpha$ (1,3)fucosyltransferase [ $\alpha$ (1,3)FT] or  $\alpha$ (1,4)fucosyltransferase [ $\alpha$ (1,4)FT] molecules or that otherwise determine  $\alpha$ (1,3)FT or  $\alpha$ (1,4)FT expression, without the need to first purify the enzyme. This system instead exploits existing reagents that allow detection of the cell surface-expressed oligosaccharide product of these enzymes, and that provide for specific assay of their enzymatic activity.

This approach requires a recipient host cell with specific properties that allow selection of the appropriate cloned cDNA molecules. The host must not express  $\alpha$ (1,3)FTs, nor cognate surface Gal  $\beta$ (1,4)[Fuc $\alpha$ (1,3)]GlcNAC linkages (SSEA-1 structures). However, this host must synthesize the appropriate substrates for surface display of SSEA-1 molecules. These substrates include the nucleotide sugar GDP-fucose, and surface-expressed glycoconjugate molecules that may serve as oligosaccharide acceptors for the transglycosylation reaction. Each of these properties are fulfilled by COS-1 cells.

Fluorescence-activated cell sorter (FACS) analysis indicated that COS-1 cells do not express surface-localized SSEA-1 determinants. Enzyme analyses performed with COS-1 extracts confirmed that absence of SSEA-1 expression was due to a deficiency of  $\alpha(1,3)$ FT activity. The inventor expected that COS-1 cells would contain substrate levels of GDP-fucose within their Golgi, since with the exception of certain lectin-resistant mutant cells lines, virtually all mammalian cells synthesize GDP-fucose and translocate it into the Golgi lumen. COS-1 cells also construct surface-expressed glycoconjugates containing unsubstituted polylactosamine moieties that represent potential oligosaccharide acceptors for  $\alpha(1,3)$ FT activity determined by a transfected cDNA. The inventor confirmed that these substrates are available for use in the construction of surface-expressed, terminal fucose linkages by demonstrating expression of a different terminally-linked fucose linkage (H  $\text{Fu}\alpha(1,2)\text{Gal}$ ) on COS-1 cells after transfection with a cloned human gene fragment that the inventor had previously shown to determine expression of an  $\alpha(1,2)$ FT. The inventor therefore observed that COS-1 cells could construct surface-expressed SSEA-1 molecules following transfection with  $\alpha(1,3)$ FT-determining cDNAs.

Isolation of a Cloned cDNA That Determines Expression of an  $\alpha(1,3)$ FT and Surface-Localized SSEA-1 Structures

The human A431 cell line has been shown to express cell surface Lewis blood group a and b structures that represent the products of an  $\alpha(1,4)$ FT. Enzyme assays performed with A431 extracts confirmed that cells also express a corresponding  $\alpha(1,3)$ FT activity. A cDNA library was therefore constructed with A431 cell mRNA in the mammalian expression vector pCDM7 and was transfected into COS-1 cells. The transfected cells were screened by the procedure of Seed using a monoclonal antibody specific for SSEA-1 determinants.

In order to follow enrichment for an  $\alpha(1,3)$ FT-determining cDNA during the selection procedure, an assay was employed in which 2'-fucosyllactose was used as an acceptor substrate. This acceptor can discriminate between the Lewis  $\alpha(1,3/1,4)$ FT and nonallelic human  $\alpha(1,3)$ FTs, since it is used efficiently by the former enzyme but not by the latter. With this assay, the inventor was unable to detect any enzyme activity in COS-1 cells transfected with the A431 cDNA library, or in cells transfected with amplified plasmid DNA isolated from the initial selection. However, amplified plasmid DNA obtained from the second selection was found to direct a low level of enzyme activity when transfected into COS-1 cells. A modest increment in enzyme activity was obtained after a third selection by panning. At this stage, "sib selection" was employed to identify and isolate a cloned  $\alpha(1,3)$ FT cDNA. Pools of clones isolated from the third panning selection were tested for their ability to generate  $\alpha(1,3)$ FT activity in



transfected COS-1 cells. From these experiments, it was estimated that approximately one in 500 clones represented a plasmid that determined  $\alpha(1,3)$ FT expression. One "active" pool of approximately 400 clones was further subdivided and the resulting pools were tested for their ability to generate enzyme activity in transfected cells. One clone (pCDM7- $\alpha(1,3/1,4)$ FT) in an active 16 clone pool was found to direct very high level expression of the  $\alpha(1,3)$ FT. FACS analysis was used to confirm that this plasmid also directs surface expression of SSEA-1 (Lewis x) determinants (Fig. 8). COS-1 cells transfected with this plasmid stain brightly with anti-SSEA-1 antibody, but not with a control IgM anti-H antibody, whereas cells transfected with pCDM7 vector alone exhibit background staining with both antibodies. Identical results were obtained in experiments where the transfected cells were stained with an anti-Lewis a antibody (Fig. 8).

Deduced Protein Sequence of the Cloned cDNA Predicts a Transmembrane Topology

The cDNA insert in pCDM7- $\alpha(1,3/1,4)$ FT (SEQ ID NO:1) is 2022 nucleotides in length, and consists of a 72 bp 5' untranslated region, a continuous open reading frame of 1083 bp, and a 3' untranslated region of 867 bp that is terminated by a poly(A) tail.

This cloned cDNA hybridizes to a single prominent 2.3 kb transcript in A431 cells showing that this insert is

essentially full-length. The nature of an additional faint 7.5 kb transcript is at present undefined. The initiation codon at the beginning of the long open reading frame is embedded within a sequence similar to the Kozak consensus initiation sequence and is preceded by two in-frame stop codons. There is also a single additional ATG upstream from the assigned initiator. This ATG also fits the Kozak consensus sequence, but initiates a very short in-frame sequence. The long open reading frame predicts a 361 amino acid protein (SEQ ID NO:2) of Mr 42,069 Da. Sequence comparisons with the latest DNA and protein sequence databases (Protein Identification Resource, Release 21.0, and GenBank, Release 60.0) identified no sequences with significant similarity to this sequence, except for a segment within the 3' untranslated region that is similar to human Alu sequences. The 3' untranslated region also contains 20 degenerate copies of a 16 nucleotide sequence of unknown functional significance.

Comparisons between the sequence predicted by the insert in pCDM7- $\alpha$ (1,3/1,4)FT and four different cloned mammalian glycosyltransferases revealed no obvious primary sequence similarities. While these latter enzymes also share no extensive primary sequence similarities, they exhibit an analogous overall structural organization. Specifically, these enzymes are representative of Type II transmembrane proteins, each composed of a short, NH<sub>2</sub>-terminal cytoplasmic

domain, a single transmembrane segment, and a larger, COOH-terminal catalytic domain that ultimately inhabits the Golgi lumen. Inspection and hydropathy analysis of the predicted protein sequence suggested that this protein maintains a similar structural organization. There is a single hydrophobic segment near the amino terminus that is comprised of 19 amino acids and is flanked by basic residues. This putative signal-anchor sequence would place 327 amino acids within the Golgi lumen, while leaving 15 residues within the cytosolic compartment.

The Protein Encoded by pCDM7- $\alpha$ (1,3/1,4)FT is a  
Fucosyltransferase

Expression data and the predicted topological similarity of this sequence to other glycosyltransferases, show that this cDNA encodes an  $\alpha$ (1,3)FT. Nonetheless, these data are also formally consistent with the possibility that this cDNA sequence instead encodes a molecule that trans-determines  $\alpha$ (1,3)FT activity by interaction with an endogenous gene, transcript, or protein. To demonstrate that enzymatic activity is directly associated with this molecule, the putative catalytic domain of the predicted polypeptide (residues 43-361 of SEQ ID NO:2) was fused to a secreted form of the IgG binding domain of Staphylococcus aureus protein A in the mammalian expression vector pPROTA, to generate the vector pPROTA- $\alpha$ (1,3/1,4-Ft)<sub>c</sub>. Since this fusion protein would

lack the putative transmembrane anchoring segment of the fucosyltransferase, the inventor expected it would be synthesized as a secreted molecule that could be affinity-purified on an IgG-containing matrix and subsequently tested for  $\alpha(1,3)$ FT activity. COS-1 cells transfected with the control vectors pCDM7 or PROTA generated no detectable cell-associated or released enzyme activity. However, conditioned media prepared from COS-1 cells transfected with pPROTA- $\alpha(1,3/1,4)$ FT<sub>c</sub> or with pCDM7- $\alpha(1,3/1,4)$ FT, contained significant quantities of  $\alpha(1,3)$ FT activity. Significantly, virtually 100% of the released activity generated by pPROTA- $\alpha(1,3/1,4)$ FT<sub>c</sub> was specifically retained by the IgG-Sepharose matrix, and approximately 24% of this activity could be recovered from the matrix after exhaustive washing. By contrast, the released activity generated by pCDM7- $\alpha(1,3/1,4)$ FT did not interact with the affinity adsorbent. These results indicate that the protein encoded by this cloned cDNA encodes a fucosyltransferase, demonstrate that information sufficient to generate  $\alpha(1,3)$ FT activity resides within the enzyme's COOH-terminal 319 amino acids, and show that this approach can be used to affinity purify the catalytic domain in an enzymatically active state as a portion of a bipartite fusion protein.

The Fucosyltransferase is a Glycosylated Transmembrane Protein

In order to confirm the transmembrane topology predicted for the enzyme, fucosyltransferase cRNA was prepared and was subjected to analyses by a series of in vitro translation experiments. The  $^{35}\text{S}$ -methionine-labelled primary translation product generated in these experiments migrated with a molecular weight of approximately 37,500 Da. The discrepancy between this observed molecular weight and the predicted one (42,069 <sup>Da</sup> ~~DA~~) may be reconciled by the observation that membrane-spanning proteins often migrate in an anomalously rapid manner through SDS-polyacrylamide gels, relative to soluble protein molecular weight markers. When this radiolabelled protein was generated by in vitro translation in the presence of canine pancreatic microsomes, it migrated with an Mr of approximately 42,000 Da. The ~ 6,000 Da increase in molecular mass observed when the translations were performed in the presence of microsomes suggests that two core glycosylation structures are added by microsomal oligosaccharyltransferase to the two potential asparagine-linked glycosylation sites during cotranslational translocation across the microsomal membrane. This product also co-sedimented with the microsomes, suggesting that the protein had become cotranslationally inserted within, or translocated across, the microsomal membrane. When this radiolabelled, microsome-associated protein was subjected to limit digestion with endoglycosidase H, its molecular mass was

reduced to a one essentially identical to that of the primary translation product. Partial endoglycosidase H digestion generated an additional band of intermediate size, that likely consists of molecules that contain a single residual core glycosylation unit. These results indicate that the addition of core oligosaccharide structures is responsible for the increase in size of the protein observed in the co-translation experiments. These observations indicate that the two potential N-glycosylation sites found within the predicted fucosyltransferase amino acid sequence are glycosylated during translocation across the microsomal membrane.

Additional support for the predicted transmembrane topology of the fucosyltransferase was provided by the results of protease protection experiments. Co-translation in the presence of microsomes yields a 42,000 Da polypeptide that is resistant to digestion with protease. The protease-digested product migrates slightly faster than the undigested, microsome-associated polypeptide; this difference is most likely accounted for by removal of some or all of the 15 NH<sub>2</sub>-terminal amino acids predicted to be displayed on the exterior of the microsomes. Addition of microsomes after translation yielded a protease-sensitive, nonglycosylated radiolabelled product, indicating that membrane insertion of the protein is a cotranslational, but not post-translation, event. A small amount of a ~ 34KDa polypeptide that is protease-sensitive and glycosylated can also be identified in these experiments. The

precise nature of this protein is unknown, but it appears in a proteinase K concentration-dependent manner. The inventor therefore suspected that it represents a proteolytic fragment of the intact protein generated when the integrity of some microsomal vesicles is disrupted. In aggregate, these experiments indicate that the bulk of this polypeptide can be sequestered within the microsomal lumen by a cotranslational translocation process, ultimately yielding a product that is N-glycosylated. These results are consistent with the type II transmembrane topology predicted by the fucosyltransferase sequence.

#### The Fucosyltransferase Can Construct Two Distinct Glycosidic Linkages

It is believed that, in general, each glycosyltransferase is competent to perform a single transglycosylation reaction, that in turn generates a single glycosidic linkage. However, genetic and biochemical studies indicate that the human Lewis blood group locus may determine expression of a single fucosyltransferase capable of generating subterminal  $\text{Fu}\alpha(1,3)$  and  $\text{Fu}\alpha(1,4)$  linkages on several type I and type II acceptor substrates. In particular, the Lewis enzyme is thought to be the only human fucosyltransferase capable of using the acceptor 2'-fucosyllactose. Since plasmid pCDM7- $\alpha(1,3/1,4)$ FT was isolated with an enrichment scheme involving an enzymatic assay based upon this acceptor substrate, it therefore seemed

likely that its cDNA insert encodes the Lewis enzyme. To confirm this, the inventor performed a series of analyses to determine the acceptor specificities of the recombinant enzyme.

Extracts of COS-1 cells transfected with pCDM7- $\alpha$ (1,3/1,4)FT were tested for their ability to catalyze transglycosylations between GDP-[ $^{14}$ C]fucose and the type I acceptor lacto-N-biose I, or the type II acceptors lactose and N-acetyllactosamine. There are only two possible classes of monofucosylated products that can be formed from each of these acceptors by known human fucosyltransferases. These are H-active products containing a  $\text{Fu}\alpha(1,2)$  linkage on the terminal Gal of these molecules, or Lewis x- or Lewis a-active products containing fucose linked in alpha anomeric configuration to the subterminal monosaccharide of these acceptors via either the monosaccharide's C4 hydroxyl (type I acceptor) or its C3 hydroxyl (type II acceptors). The inventor therefore fractionated the reaction products with a descending paper chromatography method that could distinguish between the two classes of reaction products possible with each acceptor, and thus allow determination of enzyme specificity.

The inventor found that lactose was utilized by the recombinant enzyme to form a radiolabelled compound with the chromatographic mobility characteristic of the Lewis x trisaccharide 3-fucosyllactose, and distinct from the other



possible product, the type II H trisaccharide. Likewise, these extracts also generated 3-fucosyl-N-acetyllactosamine when N-acetyllactosamine was used as an acceptor.

Radiolabelled fucose was quantitatively released from each product upon digestion with  $\alpha$ -fucosidase, indicating that the enzyme attaches fucose to these acceptor substrates in alpha anomeric configuration. These results are consistent with the flow cytometry observations indicating that pCDM7- $\alpha(1,3/1,4)$ FT determines expression of the Gal $\beta(1,4)$ [Fuc $\alpha(1,3)$ ]GlcNAc linkage representing the SSEA-1 determinant.

Moreover, the radiolabelled product of the type I acceptor lacto-N-biose I chromatographed with a mobility distinct from the H active standard 2'-fucosyllacto-N-biose I and consistent with its identity as the Lewis a trisaccharide 4-fucosyllacto-N-biose I. This product was also susceptible to digestion with  $\alpha$ -fucosidase. Identical results were obtained for all three disaccharide acceptors when affinity-purified protein A-fucosyltransferase was used in these experiments. Taken together, these results indicate that the recombinant fucosyltransferase can construct both Fuc $\alpha(1,3)$  and Fuc $\alpha(1,4)$  glycosidic linkages on type II and type I disaccharide acceptors, respectively.

In a complementary set of analyses, type I and type II blood group H trisaccharides were tested as acceptors for the enzyme encoded by the fucosyltransferase cDNA. Radiolabelled type I and type II H molecules were prepared by fucosylating

their disaccharide precursors at the C2 hydroxyl of their terminal galactose residues, using cell extracts containing the blood group H  $\alpha(1,2)$ -FT and GDP[ $^{14}\text{C}$ ]fucose. These HPLC-purified radiolabelled type I and type II H acceptors were then each used in reactions containing unlabelled GDP-fucose and affinity-purified fucosyltransferase activity generated by pPROTA- $\alpha(1,3/1,4)$ FT<sub>c</sub>. HPLC analysis of these reactions identified new radiolabelled compounds with chromatographic mobilities predicted for the Lewis b tetrasaccharide and the Lewis y tetrasaccharide, generated with the type I or type II acceptors, respectively. Virtually identical results were obtained with extracts prepared from COS-1 cells transfected with pCDM7- $\alpha(1,3/1,4)$ FT. Results of these experiments and similar ones, are summarized in Table 2.

In a third series of experiments, the inventor demonstrated that this enzyme can operate on "type I" or "type II" acceptors whose terminal galactose residues are substituted with sialic acid in  $\alpha(2,3)$  linkage, to generate the sialyl Lewis x and sialyl Lewis a tetrasaccharide determinants. Flow cytometry analysis of COS-1 cells transfected with pCDM7- $\alpha(1,3/1,4)$ FT and stained with a monoclonal anti-sialyl Lewis x antibody indicates that this plasmid can determine surface expression of the sialyl Lewis x antigen (Fig. 8), that is the product of  $\alpha(1,3)$ FT action on "type II" acceptors whose terminal galactose residues are

-181-

substituted with sialic acid in  $\alpha(2,3)$  linkage. Likewise, COS-1 cells transfected with pCDM7- $\alpha(1,3/1,4)$ FT and stained with a monoclonal anti-sialyl Lewis a antibody indicates that this plasmid can determine surface expression of the sialyl Lewis a antigen (Fig. 8), that is the product of  $\alpha(1,4)$ FT action on type I acceptors whose terminal galactose residues are substituted with sialic acid in  $\alpha(2,3)$  linkage. These analyses indicate that the fucosyltransferase encoded by pCDM7- $\alpha(1,3/1,4)$ FT is able to construct two distinct glycosidic linkages on the subterminal Glc or GlcNAc of type I and type II acceptors, and that this does not depend upon the  $\alpha(1,2)$ fucosylation or  $\alpha(2,3)$ sialylation status of the terminal galactose on these acceptors. These properties mirror those reported for the fucosyltransferase activities determined by human Lewis blood group locus, and confirm that a single fucosyltransferase can catalyze the formation of two distinct glycosidic linkages.

The Fucosyltransferase cDNA Identifies Human Genomic Sequences Syntenic to the Human Lewis Blood Group Locus

Genetic data indicate that the human Lewis blood group is determined by a locus on chromosome 19. The fucosyltransferase cDNA was therefore used for Southern blot analysis of a pair of human-mouse somatic cell hybrids that differ only by the presence or absence of human chromosome 19. The results indicate that at high stringency, the

fucosyltransferase cDNA identifies cross-hybridizing sequences located on chromosome 19. Taken together with the enzymatic analyses, these data strongly suggest that this cloned cDNA represents the product of the human Lewis blood group locus.

Experimental Procedures for Example III. "Isolation of a cloned human cDNA that encodes a GDP-Fuc: $\beta$ -D-Gal (1,4/1,3)-D-GlcNAc(/Glc)- $\alpha$ (1,3/1,4)-fucosyltransferase.

cDNA Library Construction. A cDNA library was prepared from poly(A)-plus RNA isolated from human A431 cells, using standard procedures and the mammalian expression vector pCDM7. pCDM7 lacks polyoma sequences present in the vector pCDM8, but is otherwise virtually identical. The library contained  $2.6 \times 10^6$  independent recombinants.

Cell Lines. Mouse 3T3-human hybrid cell lines KLEJ-47 and KLEJ-47/P-1 were obtained from Dr. Howard Green (Harvard University, Boston). Mouse 3T3 cells were from Dr. Vishva Dixit (University of Michigan, Ann Arbor). The origins of all other cell lines, and conditions for cell growth are as previously described in the literature.

Preparation of Panning Dishes. Panning dishes were prepared by first coating them with goat anti-mouse IgM, and then with

monoclonal anti-SSEA-1 antibody (ascites generously provided by D. Solter, diluted 1:1000).

cDNA Library Screening. The A431 library was screened as described previously. Plasmid DNA was rescued from transfected COS-1 cells adherent to panning dishes and introduced into the bacterial host MC1061/P3 by transformation. Transformants were grown to saturation in liquid culture under antibiotic selection, aliquots were removed for frozen storage, and the remainder of the culture was used to prepare plasmid DNA. A portion of this plasmid DNA was used for subsequent enrichment by transfection and immunoselection on anti-SSEA-1 panning dishes.

FACS Analysis. Transfected COS-1 cells were stained with the mouse IgM anti-SSEA-1 (anti-Lewis x) monoclonal antibody (1:1000 dilution of ascites) mouse monoclonal IgM anti-H or anti-Lewis a antibodies (Chembiomed, Ltd., Edmonton; 10  $\mu$ g/ml), a mouse monoclonal IgM anti-sialyl Lewis x antibody (CSLEX, P. Terasaki, 10ug/ml), or a mouse monoclonal IgG anti-sialyl Lewis a antibody (CSLEA, P. Terasaki, 1:1000 dilution of ascites). Cells were then stained with fluorescein-conjugated goat anti-mouse IgM (Sigma; 40  $\mu$ g/ml) and subjected to analysis by fluorescence activated cell sorting as described previously in the literature by the inventor.

Northern and Southern Blotting. A431 cell RNA was subjected to Northern blot analysis as previously described. The probe consisted of a 1.7 kb XhoI - XbaI fragment isolated from the 5' end of the cDNA insert in plasmid pCDM7- $\alpha$ (1,3/1,4)FT. This fragment does not contain the portion of this cDNA that exhibits sequence similarity to human Alu sequences. This probe was labelled by nick translation with  $\alpha$ [<sup>32</sup>P]dCTP to a specific activity of  $6 \times 10^8$  cpm/ $\mu$ g.

Genomic DNA was prepared and subjected to Southern blotting as described previously. Blots were subjected to a final wash for 30 minutes at 65°C in 0.1X SSC, 0.5% SDS. The probe used was identical to the one used for Northern blot analysis except that it was labelled with the random priming method.

Sequencing. The cDNA insert in plasmid pCDM7- $\alpha$ (1,3/1,4)FT was sequenced by the chain termination method using a double stranded plasmid DNA template and commercial reagents (Pharmacia). Both strands were sequenced using 17-mer or 19-mer oligonucleotide probes synthesized according to the sequence of the cDNA insert. The DNA sequence was assembled and analyzed using the Beckman Microgenie package, and the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group.

In Vitro Transcription-Translation. Plasmid pCDM7- $\alpha$ (1,3/1,4)FT DNA was linearized downstream from the cloned cDNA insert by digestion with NotI. Capped RNA transcripts were then generated from this linearized template using a T7 polymerase promoter based in vitro transcription kit (Pharmacia). Transcripts initiate from the T7 promoter proximal to the cDNA cloning site in pCDM7. RNA transcripts produced in vitro were used to program a rabbit reticulocyte lysate in vitro translation system (Promega), in the presence of  $^{35}\text{S}$ -methionine (Amersham), according to the manufacturer's instructions. Membrane-associated radiolabelled in vitro translation products, generated in the presence of canine pancreatic microsomal membranes (Promega) (cotranslation) or generated prior to the addition of microsomes (post-translational microsome addition), were isolated from the bulk of the soluble reaction components by centrifugation through a sucrose cushion (0.5 M sucrose, 10 mM Tris 7.4, 150 mM NaCl; 170,000 x g for 60 min). For endoglycosidase H digestions, pellets containing microsome-associated radiolabelled products were first resuspended in 50 mM sodium citrate pH 5.5, were made 0.2% in SDS, and were heated to 100°C for 4 minutes. Aliquots of this material were then diluted with an equal volume of water and subjected to digestion with either 10 mU or 5 mU of endoglycosidase H for 20 hrs at 37°C, in the presence of 0.1% BSA, 0.5% Triton X-100, 0.5 mM PMSF, 40  $\mu\text{g/ml}$  Bestatin, 10  $\mu\text{g/ml}$   $\alpha_2$

macroglobulin, and 30  $\mu\text{g/ml}$  of E-64. Alternatively, the pellets were resuspended in ice cold in vitro translation buffer containing 5 mM  $\text{CaCl}_2$ , and were subjected to incubation with 150  $\mu\text{g/ml}$  proteinase K, on ice for 1 hour, in the presence or absence of 1% Triton X-100. The various radiolabelled in vitro translation products were then denatured and reduced by heating to  $100^\circ\text{C}$  for 4 minutes in 62.5 mM Tris pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, and 0.02% bromphenol blue. Samples were then fractionated through SDS polyacrylamide gels, and the gels were subjected to autoradiography.

Fucosyltransferase Assays. Cultured cells were washed in PBS, harvested by scraping with a rubber policeman, washed again in PBS, and pelleted by centrifugation. Cell extracts were prepared by resuspending cell pellets in 1% Triton X-100 such that the final protein concentration in the extracts was approximately 5 mg/ml (BCA method, Pierce Chemical Co.).

Fucosyltransferase assays were performed in 50 mM MOPS pH 6.5, 25 mM  $\text{MnCl}_2$ , 10 mM L-fucose, 5 mM ATP, 3 mM GDP- $^{14}\text{C}$  fucose (specific activity of 600,000 cpm/nmol; 35,000 cpm per assay), 2.5 mM acceptor (e.g. 2'-fucosyllactose, N-acetyllactosamine, lactose or lacto-N-biose I), and up to 10  $\mu\text{l}$  of cell extract, in a final volume of 20  $\mu\text{l}$ . When determining  $\alpha(1,3)\text{FT}$  specific activities achieved during the sib selection process, and in the analysis of the protein A-fucosyltransferase fusion



protein experiments, the amount of added cell extract and incubation times were adjusted to yield (linear) reaction rates reflecting accurate specific activities. Reactions were incubated at 37°C for 2 or 6 hours, and then terminated by the addition of 20  $\mu$ l of ethanol, followed by dilution with 500  $\mu$ l of H<sub>2</sub>O. The terminated, diluted reactions were then centrifuged at 15,000 x g for 5 min. Fifty  $\mu$ l of each reaction supernatant was counted to determine total radioactivity, and 200  $\mu$ l of each was fractionated by Dowex-1 chromatography. The neutral radiolabelled material eluting from the column was then counted directly as a measure of product formation. Enzyme specific activity is defined as pmol of fucose transferred from GDP-fucose to acceptor per mg cell extract protein per hour. Neutral products were also further analyzed as described below by descending paper chromatography and by HPLC to confirm their identity. Parallel reactions were done in the absence of added acceptor to allow correction for transfer to endogenous acceptor molecules and for substrate and product hydrolysis. These control experiments indicated that less than 2.6% of the radioactivity in GDP-[<sup>14</sup>C]fucose was found as a neutral product in the absence of added acceptor, and that virtually all of this material represented [<sup>14</sup>C]fucose.

In instances where radiolabelled, H type I and H type II molecules were used as acceptors, nonradiolabelled GDP-fucose was included instead of GDP-[<sup>14</sup>C]fucose, and reactions were

allowed to proceed for 16 hours. Residual unreacted neutral radiolabelled acceptor substrate, and neutral radiolabelled product were isolated by Dowex-1 chromatography and then analyzed by HPLC.

Preparation of Radiolabelled H Type I and H Type II Acceptors.

Cell extracts containing a human  $\alpha(1,2)$ FT activity were used to synthesize radiolabelled type I H or type II H acceptor molecules. The cell extracts were prepared from mH1-12 cells, a mouse L cell transfectant containing a human DNA segment that encodes a human  $\alpha(1,2)$ FT. These extracts contain no detectable  $\alpha(1,3)$ FT activity or  $\alpha(1,4)$ FT activity.

Lacto-N-biose I (20 mM), or N-acetyllactosamine (20 mM), were incubated with 100  $\mu$ g of mH1-12 extract protein in 40  $\mu$ l of 25 mM sodium cacodylate pH 6.2 containing 3  $\mu$ M GDP-[ $^{14}$ C]fucose, for 16 hours at 37°C. Reactions were terminated by the addition of 40  $\mu$ l of ethanol followed by dilution with 200  $\mu$ l of water. Precipitated protein was removed by centrifugation at 12,000 x g for 5 minutes, and the neutral radiolabelled reaction products in the supernatant were isolated by Dowex-1 chromatography. The type I H trisaccharide molecules (lacto-N-biose I reaction) or type II H trisaccharide molecules (N-acetyllactosamine reaction) comprising the majority of the respective neutral radiolabelled materials were then purified by HPLC as described below.

Product Analysis by HPLC and Descending Paper Chromatography.

Neutral radiolabelled reaction products generated by affinity-purified protein A-fucosyltransferase fusion protein, or by pCDM7- $\alpha$ (1,3/1,4FT)-programmed COS-1 extracts, type I or type II disaccharide acceptors, and GDP-[ $^{14}\text{C}$ ]fucose (see above, Fucosyltransferase Assays) were fractionated by descending paper chromatography or by HPLC chromatography to determine their structures. Samples analyzed by HPLC were dissolved in 70% acetonitrile and applied to a Dynamax 60A (primary amine column, Rainin Instruments, 4.14 mm x 25 cm) equilibrated in acetonitrile:water (70:30). Compounds were eluted with a linear gradient of acetonitrile:water (70:30 to 40:60), in 1 hour, at a flow rate of 1 ml per minute. The eluant was monitored with a Beckman Instruments on-line radioisotope detector.

Samples analyzed by descending paper chromatography were dissolved in water and fractionated through Whatman No. 40 in phenol/isopropanol/formic acid/water (85:5:10:100, lower layer). After chromatography (Figure 6; 40 hours in panel A or 48 hours in panel B), air-dried chromatograms were cut into 1 cm strips and the radiolabelled compounds were eluted into 2 ml of water. The radioactivity in each eluate was determined by scintillation counting after mixing with 10 ml of scintillation cocktail. HPLC-purified  $^{14}\text{C}$ -labelled type I and type II H-active trisaccharide standards were prepared as

described above for the preparation of  $^{14}\text{C}$ -labelled type I and type II H-active acceptor trisaccharides.

$\alpha$ -L-Fucosidase Digestion. Neutral, HPLC-purified, radiolabelled fucosyltransferase products were subjected to  $\alpha$ -L-fucosidase digestion to confirm the alpha anomeric configuration of the attached fucose. (1,3) [ $^{14}\text{C}$ ]fucosyl-N-acetyllactosamine, (1,3) [ $^{14}\text{C}$ ]fucosyl-2'-fucosyllactose, (1,3) [ $^{14}\text{C}$ ]fucosyllactose, and (1,4) [ $^{14}\text{C}$ ]fucosyllacto-N-biose I were purified by HPLC, and aliquots of each (10,000 to 20,000 cpms) were digested with 100 mU of  $\alpha$ -L-fucosidase (E.C. 3.2.1.51, Boehringer-Mannheim) in 70  $\mu\text{l}$  of 100 mM Na citrate pH 5.5, at 37°C for 22 hrs. The reactions were desalted by Dowex column chromatography and subjected to HPLC analysis using conditions described above. The products of the digestion were identified by comparison to parallel separations of L-[ $^{14}\text{C}$ ]fucose and [ $^{14}\text{C}$ ]fucose-labelled acceptors. In each case, quantitative release of L-[ $^{14}\text{C}$ ]fucose was achieved by  $\alpha$ -L-fucosidase digestion.

pPROTA- $\alpha$ (1,3/1,4)FT Construction and Analysis. A 1344-bp SmaI-BamHI segment of the cDNA insert containing the putative fucosyltransferase catalytic domain was isolated from pCDM7- $\alpha$ (1,3/1,4)FT. This fragment was blunt-ended with the

Klenow fragment of DNA polymerase I, and the ends were ligated to kinased double stranded linkers (5' CGGAATTCCG 3'). The ligated fragment was gel purified, digested with EcoRI, and gel purified again. This fragment was inserted at the unique EcoRI site of PPROTA. One plasmid (ppROTA- $\alpha$ (1,3/1,4)FT<sub>c</sub>) containing a single insert in the appropriate orientation was analyzed by DNA sequencing to confirm the predicted sequence across the junctions between the vector, linker, and insert.

Plasmids pPROTA- $\alpha$ (1,3/1,4)FT<sub>c</sub>, pCDM7- $\alpha$ (1,3/1,4)FT, or pPROTA, (50  $\mu$ g each) were separately introduced into COS-1 cells (500,000 per 100 mm dish) by DEAE-dextranmediated transfection. After a 72-hour expression period, the media (10 ml) was harvested from each plate and subjected to low speed (300 X G for 8 min) and high speed (100,000 X G for 1 h) centrifugations. The supernatants were then adjusted to 0.05% Tween 20 and were either assayed directly, or were used in IgG-Sepharose binding studies. IgG-Sepharose or Sepharose 6B were preequilibrated as described by the manufacturer (Pharmacia), and then equilibrated in 10% fetal calf serum in Dulbecco's modified Eagle's medium (FCS/DMEM). Aliquots of processed supernatants containing known amounts of enzyme activity prepared from transfected COS-1 cells were incubated batchwise with 100  $\mu$ l of equilibrated IgG-Sepharose or Sepharose 6B, overnight at 4°C. Supernatants were saved for assay ("Flow thru" activity). The matrices were then washed by centrifugation, 9 times with 1 ml of 50 mM Tris pH 7.5, 1

mg/ml bovine serum albumin, twice with 1 ml of 20 mM Tris pH 7.5, 5 mM  $\text{CaCl}_2$ , 0.05% Tween-20, and once with FCS/DMEM. The matrices were then resuspended in an equal volume of FCS/DMEM. This suspension was used directly for assay of  $\alpha(1,3)\text{FT}$  activity.

Example IV. Cloning by cross-hybridization, and expression, of a DNA sequence encoding GDP-FuC: $\beta$ -D-Gal(1,4)-D-GlcNAc  $\alpha(1,3)$ fucosyltransferase (Fuc-TIV) (DNA SEQ ID NO:7, Protein SEQ ID NO:8).

The inventor had previously used a mammalian gene transfer procedure to isolate a clone cDNA (SEQ ID NO:1) that encodes the human Lewis blood group fucosyltransferase (SEQ ID NO:2). The inventor was aware of biochemical and genetic data indicating that the human genome contains two or more other structural genes that encode fucosyltransferases competent to construct surface localized Lewis x determinants ( $\text{Gal}\beta 1\rightarrow 4[\text{Fu}\alpha(1\rightarrow 3)]\text{GlcNAc-}$ ). These other enzyme(s) were thought to be polypeptides distinct from the Lewis fucosyltransferase because they exhibit different acceptor substrate specificities and differential sensitivities to divalent cation and N-ethylmaleimide inactivation. Moreover, their expression is determined by loci distinct from the Lewis blood group fucosyltransferase locus, and they display tissue specific patterns that are different from expression patterns

determined by the Lewis locus. Because these enzymes exhibit properties that are very similar to the Lewis blood group fucosyltransferase, the inventor considered it possible that their corresponding genes might be sufficiently related at the primary sequence level to be able to isolate them by cross-hybridization approaches. He considered this even though he and others had previously shown that glycosyltransferase sequences that use the same substrates are not at all related in their primary nucleic acid or amino acid sequences, since he knew that the fucosyltransferases exhibited very similar substrate requirements, and in each case constructed one or more oligosaccharide products identical to those made by the Lewis fucosyltransferase.

In consideration of the possibility that these  $\alpha(1,3)$ fucosyltransferases might be encoded by a family of structurally-related genes, he sought to isolate other such members by cross-hybridization methods, using the cloned Lewis fucosyltransferase cDNA. Low stringency Southern blot hybridization experiments indicate that the coding region of the Lewis  $\alpha(1,3)$ fucosyltransferase cDNA detects strongly hybridizing human DNA restriction fragments, as well as several weakly hybridizing fragments. Weakly hybridizing fragments were always detected regardless of the restriction enzyme used, suggesting that these represented one or more DNA sequences distinct from the authentic Lewis gene presumably represented by the strongly-hybridizing fragments. To further

examine the molecular nature of these sequences, he screened a human lambda phage genomic DNA library at low stringency with the Lewis cDNA probe. A total of 18 phages were isolated from phages representing approximately five human genomic equivalents. Southern blot analysis of 16 of these phages allowed them to be placed into three groups, based upon their restriction patterns and hybridization signal intensity strengths. Six phages representing a class of intermediate hybridization intensity were identified. A 3.6 kb cross-hybridizing *Pst*I restriction fragment was subcloned from a representative phage of this class. To determine the relationship of this fragment to cross-hybridizing fragments detected in human genomic DNA with the Lewis probe, a 400 bp *Ava*II-*Pvu*II segment of this fragment, that cross-hybridized with the Lewis coding sequence probe, was also used to probe Southern blots at low stringency. For each enzyme used to generate the Southern blots, the *Ava*II-*Pvu*II probe detected one strongly hybridizing fragment, and one or more weakly hybridizing fragments. Each strongly hybridizing fragment corresponded to one of the weakly hybridizing fragments generated by the same enzyme and detected by the Lewis probe. Likewise, the strongly hybridizing fragments detected with the Lewis probe correspond to fragments that exhibit weak hybridization to the *Ava*II-*Pvu*II probe. These results suggested that this probe, and the 3.6 kb fragment from which it was derived, represented the weakly cross-hybridizing DNA



sequences detected by the Lewis probe on genomic DNA Southern blots.

The homologous DNA restriction fragment maintains a single long open reading frame that predicts a polypeptide with similarity to the Lewis blood group  $\alpha(1,3/1,4)$  fucosyltransferase cDNA. DNA sequence analysis of the 3.6 kb PstI fragment (SEQ ID NO:7) identified a single long open reading frame within its 3' portion corresponding to sequences that cross-hybridized to the Lewis cDNA probe (Fig. 4 and Fig. 5). This reading frame begins with a methionine codon that is found within a sequence context consistent with Kozak's consensus rules for mammalian translation initiation. Moreover, hydropathy analysis of the protein sequence predicted by this reading frame indicates a single hydrophobic segment at its NH<sub>2</sub>-terminus, suggesting that the predicted polypeptide (SEQ ID NO:8) would maintain the type II transmembrane orientation typical of mammalian glycosyltransferases. The distal portion of this reading frame shares a substantial amount of amino acid sequence identity with the corresponding portion of the Lewis fucosyltransferase (Fig. 5). These sequences share the highest degree of similarity between their COOH-terminal portions, within the catalytic domain of the Lewis fucosyltransferase. Sequence divergence occurs toward the predicted NH<sub>2</sub>-end, within the "stem" and transmembrane regions of the latter enzyme.

The DNA restriction fragment detects mRNA transcripts in HL-60 myeloid cells. To test the possibility that this segment represents a functional  $\alpha(1,3)$ fucosyltransferase gene, a portion of it was used as a probe to identify transcripts in a cell line known to express such enzymes. The HL-60 human cell line was examined since these myeloid lineage cells are known to express one or more  $\alpha(1,3)$ fucosyltransferases that are distinct from the Lewis  $\alpha(1,3)$ fucosyltransferase. Northern blot analysis of polyadenylated mRNA isolated from these cells, using the 400 bp *Av*II-*Pvu*II segment corresponding to a portion of the open reading frame, identified four distinct transcripts. By contrast, no transcripts were detected when the same analysis was performed using the Lewis cDNA. These results are consistent with the possibility that the fucosyltransferase(s) expressed by HL-60 cells are encoded by the open reading frame in the cloned *Pst*I segment.

The open reading frame in the homologous DNA restriction fragment determines expression of an  $\alpha(1,3)$ fucosyltransferase. To determine if this segment encodes an  $\alpha(1,3)$ fucosyltransferase, the 3.6 kb *Pst*I fragment was cloned into a mammalian expression vector and the resulting plasmid (pCDNA1-Fuc-TIV, "Experimental Procedures") was introduced into two types of mammalian host cells by transfection. Transfected cells were then analyzed for vector-dependent cell surface glycoconjugate expression and for fucosyltransferase

activity. COS-1 cells and CHO cells were used as hosts for these experiments since neither cell line normally expresses any detectable  $\alpha(1,3)$ - and  $\alpha(1,4)$ fucosyltransferase activities. Likewise, COS-1 and CHO cells do not normally express detectable amounts of cell surface  $\text{Gal}\beta 1\rightarrow 4[\text{Fu}\alpha(1\rightarrow 3)]\text{GlcNAc}-(\text{Lewis x}, \text{SSEA-1})$  moieties, or the  $\alpha 2\rightarrow 3$  sialylated derivative ( $\text{NeuAc}\alpha 2\rightarrow 3\text{Gal}\beta 1\rightarrow 4[\text{Fu}\alpha(1\rightarrow 3)]\text{GlcNAc}-$ , sialyl Lewis x). These cells do, however maintain surface-display of the non-fucosylated neutral and  $\alpha 2\rightarrow 3$ -sialylated type II oligosaccharides that can function as precursors to such molecules, via the action of the  $\alpha(1,3)$ fucosyltransferase encoded by a transfected Lewis cDNA expression vector. COS-1 cells also maintain surface display of the type I precursors to the Lewis a ( $\text{Gal}\beta 1\rightarrow 3[\text{Fu}\alpha(1\rightarrow 4)]\text{GlcNAc}-$ ) and sialyl Lewis a ( $\text{NeuNAc}\alpha 2\rightarrow 3\text{Gal}\beta 1\rightarrow 3[\text{Fu}\alpha(1\rightarrow 4)]\text{GlcNAc}-$ ) moieties. The vector pCDNAI was used since this plasmid efficiently transcribes exogenous, subcloned sequences in mammalian hosts by virtue of the cytomegalovirus immediate early promoter sequences in the vector.

In initial biochemical analyses, extracts prepared from COS-1 cells transfected with plasmid pCDNAI-Fuc-TIV were tested for the presence of vector-dependent fucosyltransferase activity, using several low molecular weight acceptor substrates. In a standard fucosyltransferase assay ("Experimental Procedures"), extracts prepared from

pCDNA1-Fuc-TIV transfected cells, but not from control transfectants, contained a fucosyltransferase activity (296 pmol/mg-h) that utilized the type II disaccharide acceptor N-acetyllactosamine to yield a radiolabeled product with a chromatographic mobility ("Experimental Procedures") characteristic of authentic  $\text{Gal}\beta 1\text{-}3[\text{Fu}\alpha(1\text{-}4)]\text{GlcNAc}$  ( $R_{2'}\text{-fucosyl-N-acetyllactosamine} = 0.85$ ). However, under these assay conditions, two other neutral type II molecules (2'-fucosyllactose, lactose) did not function as efficiently as N-acetyllactosamine as acceptor substrates for the fucosyltransferase in these extracts (17 and 10 pmol/mg-h, respectively, for 2'-fucosyllactose and lactose). Only a trace amount of transfer could be detected using the type I substrate lacto-N-biose I.

Likewise, the inventor did not detect fucose transfer to the sialylated acceptor  $\text{NeuAc}\alpha(2\text{-}3)\text{Gal}\beta(1\text{-}4)\text{GlcNAc}$  (less than 1 pmol/mg-h), even in extracts that exhibited a relatively large amount of activity toward N-acetyllactosamine (474 pmol/mg-h). By contrast, under these same conditions, extracts containing the Lewis blood group fucosyltransferase utilized both the sialylated acceptor (297 pmol/mg-h) and N-acetyllactosamine (526 pmol/mg-h), to form, respectively, the sialyl Lewis x tetrasaccharide and the Lewis x trisaccharide (see "Experimental Procedures"). Thus, the restricted acceptor preference exhibited by this enzyme *in vitro* contrasts remarkably with that exhibited by the Lewis

$\alpha(1,3/1,4)$ fucosyltransferase, which can efficiently utilize each of the five acceptors tested. These results are summarized in Table 2.

COS-1 cells transfected with pCDNA1-Fuc-TIV were also analyzed by flow cytometry to detect *de novo*, vector-dependent surface expression of these oligosaccharide products, to allow an assessment of the enzyme's *in vivo* acceptor substrate requirements. The transfected COS-1 cells exhibited positive staining with a monoclonal antibody directed against the Lewis x moiety  $\text{Gal}\beta 1\rightarrow 4[\text{Fu}\alpha(1\rightarrow 3)]\text{GlcNAc-}$  (Fig. 8), whereas cells transfected with the pCDNA1 vector without insert did not express this determinant. However, COS-1 cells transfected with pCDNA1-Fuc-TIV, or with its control plasmid, did not stain with antibodies specific for the sialyl Lewis x antigen (Fig. 8). Likewise, the transfected cells did not exhibit detectable surface expression of Lewis a or sialyl Lewis a molecules (Fig. 8).

Polylactosaminoglycans with terminal  $\alpha(2\rightarrow 3)$ -linked sialic acid also exist that maintain a single internal  $\alpha(1,3)$ -linked fucose on the N-acetylglucosamine residue of the penultimate lactosamine repeat. This determinant ( $\text{NeuAc}\alpha 2\rightarrow 3\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 3\text{Gal}\beta 1\rightarrow 4[\text{Fu}\alpha(1\rightarrow 3)]\text{GlcNAc-}$ ) can be detected on the surfaces of myeloid cells by the monoclonal antibody VIM-2, and may be constructed by the action of  $\alpha(1,3)$ fucosyltransferase(s) on type II polylactosamine acceptors whose terminal galactose residues are substituted

with  $\alpha(2,3)$ sialic acid moieties. Neither COS-1 cells transfected with the Lewis  $\alpha(1,3/1,4)$ fucosyltransferase, nor COS-1 cells transfected with plasmid pCDNA1-Fuc-TIV display detectable amounts of this determinant.

Virtually identical results were obtained with COS-1 cells transfected with the plasmid pCDNAI- $\alpha(1,3)$ FTM<sub>1u</sub> ("Experimental Procedures"). This vector encompasses sequences corresponding to bp-1904 through the end of the open reading frame in Fig. 4. These results provide additional evidence for the hypothesis that the open reading frame displayed in Fig. 4 corresponds to the coding portion of this fucosyltransferase gene.

To further demonstrate that enzymatic activity is directly associated with this protein, the putative catalytic domain of the predicted polypeptide (amino acids 50 to 405 of SEQ ID NO: 8) was fused to a secreted form of the IgG binding domain of *Staphylococcus aureus* protein A in the mammalian expression vector pPROTA, to generate the vector pPROTA- $\alpha$ (Fuc-TIV)<sub>c</sub>. Since this fusion protein would lack the putative transmembrane anchoring segment of the fucosyltransferase, the inventor expected it would be synthesized as a secreted molecule that could be affinity-purified on an IgG-containing matrix and subsequently tested for  $\alpha(1,3)$ FT activity. COS-1 cells transfected with the control vectors pCDM7 or pPROTA generated no detectable cell-associated or released enzyme activity. However, conditioned media prepared from COS-1

cells transfected with pPROTA- $\alpha$ (Fuc-TIV)<sub>c</sub> contained significant quantities of  $\alpha$ (1,3)FT activity when assayed with N-acetyllactosamine. Virtually 100% of the released activity generated by pPROTA- $\alpha$ (Fuc-TIV)<sub>c</sub> is specifically retained by the IgG-Sepharose matrix. These results indicate that the protein encoded by this cloned DNA segment encodes a fucosyltransferase, and demonstrate that information sufficient to generate  $\alpha$ (1,3)FT activity resides within the enzyme's COOH-terminal 356 amino acids.

Biochemical analysis of extracts prepared from a CHO cell line transfected with pCDNA1-Fuc-TIV (CHO-FT3 cells) yielded results similar to those obtained with the transfected COS-1 cells. In the standard fucosyltransferase assay (Experimental Procedures), extracts prepared from the control transfected cell line CHO-V did not contain  $\alpha$ (1,3)fucosyltransferase activity when tested with N-acetyllactosamine, 2'-fucosyllactose, lactose, or lacto-N-biose I, or NeuAc $\alpha$ (2 $\rightarrow$ 3)Gal(1 $\rightarrow$ 4)GlcNAc. By contrast, extracts prepared from the CHO-FT3 line contained an  $\alpha$ (1,3)fucosyltransferase activity (59.1 pmol/mg-h) that utilized the type II disaccharide acceptor N-acetyllactosamine to yield a radiolabeled product characteristic of authentic Gal $\beta$ (1 $\rightarrow$ 4)[Fuc $\alpha$ (1 $\rightarrow$ 3)]GlcNAc (R 2'-fucosyl-N-acetyllactosamine = 0.85) (see "Experimental Procedures"). Under these assay conditions, the CHO-FT3 extracts utilized the type II acceptor molecules 2'-fucosyllactose and lactose with substantially

lower efficiency (5.8 pmol/mg-h and 2.0 pmol/mg-h, respectively). Virtually no transfer could be detected when these extracts were tested with the type I substrate lacto-N-biose I (<1 pmol/mg-h) or with the sialyl Lewis x precursor NeuNAc $\alpha$ 2 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4GlcNAc (<1 pmol/mg-h). These results confirm those obtained with extracts prepared from the transfected COS-1 cells, and indicate that, to a first approximation, the COS-1 and CHO genetic backgrounds do not strongly influence the enzyme's ability to utilize these five low molecular weight acceptor substrates.

With one striking and important exception, flow cytometry analyses with the CHO-FT3 cells were virtually identical to those obtained with the transfected COS-1 cells. CHO-FT3 cells exhibit uniform, bright staining with anti-Lewis x antibody, but not with antibody directed against the sialyl Lewis x molecule. Control transfected cells do not stain with either antibody. As expected, neither cell line stained with antibodies against the neutral and  $\alpha$ 2 $\rightarrow$ 3-sialylated Lewis a isomers, since CHO cells do not construct type I precursors. However, these cells differed in an important way from the transfected COS-1 cells, in that, like CHO cells transfected with the Lewis  $\alpha$ (1,3/1,4)fucosyltransferase cDNA (pCDM7- $\alpha$ (1,3/1,4)FT), these cells expressed substantial amounts of the VIM-2 determinant.

Taken together with the results of the biochemical analyses performed with extracts from the transfected cells



and summarized in Table 2, the flow cytometry analyses presented in Fig. 8, the protein A gene fusion experiments, and the DNA sequence analysis indicate that plasmid pCDNA1-Fuc-TIV encodes an  $\alpha(1,3)$ fucosyltransferase. Transfection results obtained with plasmid pCDNA1- $\alpha(1,3)$ FTM1u also demonstrate that this enzyme is encoded by the open reading frame displayed in Fig. 4. The results further indicate that this enzyme can utilize type II precursors, but not type I precursors, and suggest that the enzyme cannot efficiently utilize  $\alpha 2 \rightarrow 3$ -sialylated type II glycoconjugates to form the sialyl Lewis x determinant.

Experimental Procedures for Example IV, "Cloning by cross-hybridization, and expression, of a DNA sequence encoding GDP-Fuc: $\beta$ -D-Gal(1.4)1-D-GlcNAc  $\alpha(1,3)$ -Fucosyltransferase: (Fuc-TIV)"

Cell culture. The source and growth conditions of COS-1 cells, CHO transfectants, and A431 cells are as previously described (Ernst et al, J. Biol. Chem. (1989) 265:3436-3447; Rajan et al, J. Biol. Chem. (1989) 264:11158-11167). The human HL-60 cell line was obtained from Dr. Steve Kunkel (University of Michigan, Ann Arbor). HL-60 cells were grown in 10% fetal calf serum and Dulbeccos Modified Eagle's Medium.

Antibodies. The anti-Lex antibody anti-SSEA-1 Solter et al, Proc. Nat. Acad. Sci. (USA) (1978) 75:5565-5569) (mouse

monoclonal IgM as ascites) was used. Anti-H and anti-Lewis a antibodies (mouse monoclonal IgM, antigen affinity purified) were purchased from Chembiomed Ltd. (Edmonton, Alberta). Anti-sialyl Lewis x antibody CSLEX1 (Fukushima et al, Cancer Res. (1984) 44:5279-5285) (mouse monoclonal IgM, HPLC purified) and anti-sialyl Lewis a antibody CSLEA1 (Chia et al, Cancer Res. (1985) 45:435-437) (mouse monoclonal IgG3, ammonium sulfate precipitate) were used. Anti-VIM-2 was obtained from Dr. Bruce Macher (San Francisco State University). A pooled mouse IgG antibody preparation (MsIg) was purchased from Coulter. Fluorescein-conjugated goat anti-mouse IgM or IgG antibodies were purchased from Sigma.

Human genomic library construction. High molecular weight human genomic DNA was prepared from peripheral blood leukocytes as described previously (Ernst et al (1989)). Genomic DNA was subjected to partial digestion with the restriction endonuclease Sau3A. The partially digested genomic DNA was size fractionated by ultracentrifugation through a sodium chloride gradient. Fractions enriched for DNA fragments between 8 Kb and 20 Kb were ligated to XhoI digested lambda FIX (Stratagene) phage arms that had been partially filled in with dTTP and dCTP to make the ends compatible with the Sau3A fragments. The ligation mixture was packaged in vitro with commercial packaging extracts (Stratagene), titered on E. coli host TAP90 (Patteron et al, Nucl. Acids Res. (1987) 15:6298). Approximately  $1.0 \times 10^6$

recombinant lambda phage were screened by plaque hybridization. Plaque lifts were prepared using nitrocellulose filters (Schleicher and Schuell) and were prehybridized at 42°C for 16 hours in 50% formamide, 5X SSC, 10X Denhart's solution, and 0.1% SDS. Filters were hybridized for 72 hours at 35°C in prehybridization solution containing 10% dextran sulfate, and 100 micrograms per ml denatured salmon sperm DNA. The probe consisted of a 1.7 Kb XhoI-XbaI fragment isolated from the 5' end of a cDNA insert encoding the Lewis blood group  $\alpha(1,3/1,4)$  fucosyltransferase which was labeled with [ $\alpha$ -<sup>32</sup>P] dCTP. The filters were rinsed three times for 20 minutes each at room temperature in 2X SSC and then once for 40 minutes at 50°C and 1X SSC, 0.5% SDS. Filters were then subjected to autoradiography. Eighteen independent hybridization-positive plaques were identified after 2 additional cycles of plaque hybridization. Phage DNAs were prepared from liquid lysates and were subsequently characterized by restriction endonuclease digestions and Southern blot analyses.

DNA sequence analysis. Phage DNA was digested with PstI and a 3.8 Kb fragment homologous to the human  $\alpha(1,3/1,4)$  fucosyltransferase cDNA was gel purified and ligated into the PstI site of pTZ18R. A representative subclone containing a single insert was designated pFT-3. A 970 bp hybridization-positive AvaII-PstI fragment was isolated from insert in pFT-3 and subcloned into pTZ18R. The DNA sequence

of the insert in this plasmid was determined by the dideoxy chain determination method using T7 DNA polymerase (Pharmacia LKB Biotechnology, Inc.) and oligonucleotides synthesized according to flanking plasmid sequences and subsequently according to the insert sequence. This sequence data was used to generate additional synthetic deoxynucleotides which were then used to sequence portions of the insert in pFT-3. Sequence analysis was performed using the sequence analysis software package of the University of Wisconsin Genetics Computer Group.

Southern blot analysis. High molecular weight human genomic DNA was prepared from whole peripheral blood. Genomic DNA (10  $\mu$ g) was digested with restriction endonucleases, fractionated through a 0.8% agarose gel, and subjected to Southern blot analysis. To aid in the comparison of hybridization patterns obtained with different probes, duplicate blots were prepared from identical sets of restriction digests electrophoresed on a single gel. Southern blots were hybridized with the temperature being maintained at 35°C. Southern blots were probed with the 1.7 Kb XhoI-XbaI fragment of plasmid pCDM7- $\alpha$ (1,3/1,4)FT which represents the 5' end of the human cDNA encoding the Lewis  $\alpha$ (1,3/1,4)fucosyltransferase enzyme. Alternatively, Southern blots were probed with a 400 bp AvaII-PvuII fragment isolated from the insert in pFT-3. Following hybridization, blots were

rinsed twice in 2X SSC 0.5% SDS at room temperature for 10 minutes, washed, and then subjected to autoradiography.

Northern blot analysis. Total RNA was prepared from cultured cells Poly A+ RNA was then isolated from total RNA by oligo dT cellulose column chromatography using commercially supplied columns (Clontech) and procedures supplied by the manufacturer. RNA samples were electrophoresed through 1.0% agarose gels containing formaldehyde and were then transferred to a nylon tembrane (Hybond-N, Amersham). Northern blots were prehybridized for 1 hour at 61°C in 1X PE (16), 5X SSC, 1% sodium dodecyl sulfate, and 100 µg/ml sheared salmon sperm DNA. Blots were then hybridized for at least 16 hours at 61°C in the same hybridization solution. The probe was a radiolabelled 400 bp AvaII-PvuII fragment isolated from the insert in pFT-3. Following hybridization, blots were subjected to three, ten minute room temperature rinses in 2X SSC, and then washed for 30 minutes at 62°C in 2X SSC, 0.2% SDS.

Transfection and expression of the insert in pFT-3. The 3.8 Kb PstI insert in plasmid pFT-3 was excised and cloned into the PstI site in the mammalian expression plasmid pCDNA1 (Invitrogen). One plasmid with a single insert in the sense orientation with respect to the plasmid's CMV promoter enhancer sequences was designated pCDNA1-Fuc-TIV, and was used for subsequent analysis.

Construction and radiolabeling of stably transfected CHO cell lines. CHO Ade-C cells were transfected with ScaI-linearized pCDM7-Fuc-TIV, co-precipitated in a 10-fold molar excess over EcoRI-linearized pSV2-Neo. A single, clonal, SSEA-1-positive cell line (CHO-FT3) was derived from this population. Cell extracts prepared from CHO-FT3 contained substantial amounts of  $\alpha(1,3)$ fucosyltransferase activity when assayed with the acceptor N-acetyllactosamine.

FACS analysis. COS-1 cells transfected with plasmid DNAs were harvested 48-72 hours after transfection, and stained with monoclonal antibodies diluted in staining media. Anti-Lewis a and anti-H antibodies (mouse IgM monoclonal; antigen-affinity purified; Chembiomed, Edmonton) were used at 10  $\mu$ g/ml. Anti-SSEA-1 (mouse monoclonal IgM; ascites) was used at a dilution of 1:1000. Anti-sialyl Lewis x (mouse monoclonal IgM; HPLC purified from ascites) was used at 10  $\mu$ g/ml. Anti-sialyl Lewis a (mouse monoclonal IgG3; ammonium sulfate precipitate of ascites) was used at a dilution of 1:1000. Control mouse IgG3 antibody (MsIg, Coulter) was used at a concentration of 10  $\mu$ g/ml. Anti-VIM-2 antibody (mouse monoclonal IgM; ascites) was used at a dilution of 1:200. Cells were then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgM or IgG, as appropriate, and were then subjected to analysis on a FACScan (Becton-Dickinson).

Fucosyltransferase assays. Cell extracts containing 1% Triton X-100 were prepared from transfected COS-1 cells. Fucosyltransferase assays were performed in a total volume of 20  $\mu$ l, and contained 50 mM sodium cacodylate, pH 6.2, 5 mM ATP, 10 mM fucose, 20 mM  $\text{MnCl}_2$ , 3  $\mu$ M GDP- $^{14}\text{C}$ -fucose, and 5  $\mu$ l (30  $\mu$ g protein) of cell extract. Acceptor substrates were added to a final concentration of 20 mM. Reactions were incubated at 37°C for 1 hour and terminated by addition of 20  $\mu$ l ethanol, followed by addition of 600  $\mu$ l of distilled water. An aliquot of each reaction (50  $\mu$ l) was subjected to scintillation counting to determine total radioactivity in the reaction. Another aliquot (200  $\mu$ l) was applied to a column containing 400  $\mu$ l of Dowex IX2-400, formate form. The flow through fraction, and 2  $\mu$ l of a subsequent water elution, were collected and pooled, and an aliquot was subjected to scintillation counting to quantitate incorporation of radioactive fucose into neutral product. Descending paper chromatography was used to confirm the structure of the product formed with the acceptor N-acetyllactosamine. The neutral product in the Dowex column eluate was concentrated by lyophilization, resuspended in a small volume of water, and fractionated through Whatman No. 40 in phenol/isopropanol/formic acid/water (85:5:10:100, lower layer). After chromatography (40 hours), the air-dried chromatogram was cut into 1 cm strips and the strips eluted into 2 ml of water.

Radioactivity in each eluate was determined by scintillation counting after mixing with 10 ml of scintillation cocktail.

An affinity-purified, protein A-Lewis fucosyltransferase fusion protein was used to prepare an authentic, radiolabeled  $\text{Gal}\beta(1\rightarrow4)[^{14}\text{C}\text{-Fuca}(1\rightarrow3)]\text{GlcNAc}$  standard for this analysis. This fusion protein was incubated with 20 mM N-acetyllactosamine, 150  $\mu\text{M}$  GDP- $[^{14}\text{C}]$ fucose (sp. act. = 3,800 cpms/nmol), in a standard fucosyltransferase reaction mixture. The neutral, radiolabeled product was purified by amine adsorption HPLC on a Waters Carbohydrate Analysis column, using an isocratic gradient consisting of 70% acetonitrile in water, at a flow rate of 1 ml/min. The product (17 nmol) was subjected to analysis by  $^{14}\text{C}$  NMR (Center for Complex Carbohydrate Research, Athens, GA). The sample was exchanged repeatedly in  $\text{D}_2\text{O}$  and subjected to NMR analysis at 500 MHz. The proton NMR spectrum was recorded on a Bruker AM500 instrument at 28°C. Chemical shifts are relative to acetate ( $\delta$ , 1.908 ppm). The structure of the expected trisaccharide  $\text{Gal}\beta(1\rightarrow4)[\text{Fuca}(1\rightarrow3)]\text{GlcNAc}$ , was verified by 500 MHz spectroscopy. The spectrum, recorded in  $\text{D}_2\text{O}$  at 28°C, showed H-1 signals for GlcNAc ( $\alpha$ -anomer) at  $\delta\approx 5.102$ , for Gal at  $\delta\approx 4.467$  and 4.454 ppm (for the  $\alpha$ - and  $\beta$ -anomers, respectively, of the trisaccharide) and for Fuc at  $\delta\approx 5.102$  ppm. The anomeric signal for the  $\beta$ -anomer of GlcNAc was obscured by the residual HOD peak ( $\delta\approx 4.72$  ppm). The methyl signals of the GlcNAc N-acetyl group and the C6 protons of Fuc were observed



at  $\delta \approx 2.032$  and 1.178 ppm, respectively. These chemical shifts match those published for the authentic trisaccharide  $\text{Gal}\beta(1\rightarrow4)[\text{Fuca}(1\rightarrow3)]\text{GlcNAc}$ .

$\alpha$ -L-Fucosidase Digestion. The neutral, chromatographically-purified radiolabeled fucosyltransferase product was subjected to  $\alpha$ -L-fucosidase digestion to confirm the alpha anomeric configuration of the attached fucose. 3- $^{14}\text{C}$  fucosyl-N-acetyllactosamine was purified by descending paper chromatography as described above, and an aliquot (7000 cpm) was digested with 40 mU of  $\alpha$ -L-fucosidase (E.C. 3.2.1.51, Boehringer-Mannheim) in 20  $\mu\text{l}$  of 100 mM Na citrate pH 5.5, at 37°C for 22 hrs. The reaction was desalted by Dowex column chromatography and subjected to HPLC analysis using conditions described above for preparation of the radiolabeled standard. The product of the digestion was identified by comparison to parallel separations of L-  $^{14}\text{C}$  fucose and the 3- $^{14}\text{C}$  fucosyl-N-acetyllactosamine starting material. Quantitative release of L- $^{14}\text{C}$  fucose was achieved by  $\alpha$ -L-fucosidase digestion.

Example V. Isolation of a GDP-Fuc: $\beta$ -D-Gal(1,4)-D-GlcNAc  
 $\alpha$ (1,3)Fucosyltransferase (Fuc-TV, DNA SEQ ID  
NO:10, Protein SEQ ID NO:11) through  
cross-hybridization:

Molecular cloning of a human genomic DNA segment that cross-hybridizes to the Lewis blood group  $\alpha$ (1,3/1,4) fucosyltransferase cDNA: Low stringency Southern blot hybridization experiments have indicated to the inventor that the coding region of the Lewis fucosyltransferase cDNA detects strongly hybridizing restriction fragments, as well as several weakly hybridizing fragments.

The inventor expected that the strongly hybridizing fragments represented one or more genes similar to the Lewis fucosyltransferase cDNA. To further examine the molecular nature of these sequences, as noted above in Example IV, the inventor screened a human lambda phage genomic DNA library at low stringency with the Lewis cDNA probe. A total of 18 phages were isolated from phages representing approximately five human genomic equivalents. Southern blot analysis of 16 of these phages allowed them to be placed into three groups, based upon their restriction patterns and hybridization signal intensity strengths. Several phages representing a class with strong hybridization intensities were identified. Cross-hybridizing restriction fragments isolated from one of these phages was subcloned and sequenced.

The homologous DNA restriction fragment maintains a single long open reading frame that predicts a polypeptide with strong similarity to the Lewis blood group  $\alpha(1,3/1,4)$  fucosyltransferase cDNA: DNA sequence analysis of the subcloned fragments identified a single long open reading frame within its 3' portion, beginning at base pair 1 and ending at base pair 1125 (SEQ ID NO:10) (see Figure 6). This reading frame begins with a methionine codon that is found within a sequence context consistent with Kozak's consensus rules for mammalian translation initiation. A hydropathy analysis of the protein sequence predicted by this reading frame (SEQ ID NO:11) would predict a single hydrophobic segment at its  $\text{NH}_2$ -terminus, suggesting that the predicted polypeptide would maintain the type II transmembrane orientation typical of mammalian glycosyltransferases. Virtually the entire length of this reading frame shares a strikingly high amount of amino acid and nucleic acid sequence identity with the corresponding portion of the Lewis fucosyltransferase cDNA (Figure 6). This sequence similarity diverges in just a few positions, most notably at base pair 139 within the open reading frame. This 33 base pair insertion, relative to the Lewis cDNA, would create a peptide insertion of 11 amino acids, relative to the Lewis fucosyltransferase. Because of the substantial sequence similarity between these two DNA sequences, and their derived protein sequences, the inventor expected this new

cross-hybridizing sequence to represent a single exonic sequence, representing a heretofore undefined gene, that encodes a fucosyltransferase.

The homologous DNA restriction fragment determines expression of an  $\alpha(1,3)$ fucosyltransferase: To determine if this segment encodes a functional fucosyltransferase, a 1.94 kb *Eco*I-*Xba*I fragment containing the entire open reading frame was cloned into a mammalian expression vector, and the resulting plasmid (pCDNA1-Fuc-TV) was introduced into mammalian host cells by transfection. COS-1 cells were used as hosts for these experiments since these cells normally express virtually undetectable  $\alpha(1,3)$ - and  $\alpha(1,4)$ fucosyltransferase activities. Likewise, COS-1 cells do not normally express detectable amounts of cell surface  $\text{Gal}\beta 1 \rightarrow 4 (\text{Fuc}\alpha(1 \rightarrow 3))\text{GlcNAc}$ - (Lewis x, SSEA-1) or  $\text{Gal}\beta 1 \rightarrow 3 [\text{Fuc}\alpha(1 \rightarrow 4)]\text{GlcNAc}$ - (Lewis a) moieties, whereas they do maintain surface-display of non-fucosylated type II and type I oligosaccharide precursors necessary for the construction of such molecules. The vector pCDNA1 was used, since this plasmid efficiently transcribes exogenous, subcloned sequences in COS-1 hosts by virtue of the cytomegalovirus immediate early promoter sequences in the vector, and is maintained in these cells as a multicopy episome.

COS-1 cells transfected with pCDNA1-Fuc-TV were first analyzed by flow cytometry to detect *de novo*, vector-dependent

surface expression of these oligosaccharides. A substantial fraction of these transfected cells exhibited bright staining with a monoclonal antibody directed against the Lewis x moiety (Gal $\beta$ (1 $\rightarrow$ 4)[Fuc $\alpha$ (1 $\rightarrow$ 3)]GlcNAc-) (Fig.8), whereas cells transfected with the pCDNA1 vector without insert did not express this determinant. By contrast, COS-1 cells transfected with pCDNA1-Fuc-TV, or with its control plasmid, did not stain with antibodies specific for the type I-based Lewis a trisaccharide (Fig. 8).

Taken together, these results are consistent with the results of DNA sequence analysis indicating that this segment encodes an  $\alpha$ (1,3)fucosyltransferase competent to utilize neutral type II oligosaccharide precursor, but that the enzyme cannot efficiently utilize type I glycoconjugates and thus does not exhibit strong  $\alpha$ (1,4)fucosyltransferase activity.

There is evidence supporting the possibility that one or more human  $\alpha$ (1,3)fucosyltransferases exist that can utilize type II acceptors whose terminal galactose residues are substituted with  $\alpha$ (2,3)sialic acid moieties. Such enzymes can fucosylate these molecules to form the sialyl-Lewis x determinant (NeuAc $\alpha$ (2 $\rightarrow$ 3)Gal $\beta$ (1 $\rightarrow$ 4)[Fuc $\alpha$ (1 $\rightarrow$ 3)]GlcNAc-). The Lewis fucosyltransferase, for example, is competent to perform this reaction.

It was therefore of interest to determine if the fucosyltransferase apparently encoded by plasmid pCDNA1-Fuc-TV would be capable of constructing sialyl-Lewis X determinants.

COS-1 cells maintain surface-expressed glycoconjugates terminating in (NeuAc $\alpha$ (2,3)Gal $\beta$ (1,4)GlcNAc-; these represent acceptor substrates for sialyl-Lewis construction determined via the action of enzymes encoded by transfected fucosyltransferase expression vectors. COS-1 cells were therefore transfected with plasmid pCDNA1-Fuc-TV, stained with a monoclonal anti-sialyl Lewis x antibody, and subjected to flow cytometry analysis. A significant amount of staining was detected, relative to control cell transfected with the vector alone, or relative to pCDNA1- $\alpha$ (1,3)-Fuc-TV-transfected cells stained with a negative control antibody (anti-H). (Fig. 8).

However, these cells did not stain with an antibody specific for the type I-based, sialyl-Lewis a determinant (NeuAc $\alpha$ (2,3)Gal $\beta$ (1,3)[Fuca(1,4)]GlcNAc-) (Fig. 8). By contrast, the inventor had previously observed that a substantial fraction of COS-1 cells transfected with the Lewis fucosyltransferase expression plasmid pCDM7- $\alpha$ (1,3/1,4)FT exhibit bright staining with the sialyl-Lewis x and sialyl-Lewis a antibodies, as predicted by biochemical analysis of the acceptor substrate specificity of this enzyme.

The conclusion that plasmid pCDNA1-Fuc-TV encodes an  $\alpha$ (1,3)fucosyltransferase was confirmed by biochemical analysis of the acceptor substrate requirements of the enzyme in extracts of COS-1 cells transfected with plasmid pCDNA1-Fuc-TV. As expected, the enzyme in these extracts utilized the type II disaccharide acceptor N-acetyllactosmine, to yield the

predicted product  $\text{Gal}\beta(1\rightarrow4)[\text{Fu}\alpha(1\rightarrow3)]\text{GlcNAc}$ . The enzyme also efficiently utilized the trisaccharide  $\text{NeuAc}\alpha(2,3)\text{Gal}\beta(1,4)\text{GlcNAc}$  to form the sialyl Lewis x tetrasaccharide (Table 2). Under these conditions, other type II molecules, including lactose, and the  $\alpha(1,2)$  fucosylated type II acceptor  $\text{Fu}\alpha(1\rightarrow2)\text{Gal}\beta(1\rightarrow4)\text{Glc}$ , did function as acceptor substrates for the fucosyltransferase in these extracts, although with substantially lower efficiencies. (Summarized in Table 2).

Interestingly, the type I substrate lacto-N-biose I is also utilized by this enzyme, although again at low efficiency (Table 2). This suggests that the enzyme can also function as an  $\alpha(1,4)$  fucosyltransferase, but at a very low efficiency, as also suggested by the absence of  $\alpha(1,4)$  structures on flow cytometry analysis. The acceptor preferences exhibited by this enzyme contrast with that exhibited by the Lewis fucosyltransferase, which is able to efficiently utilize each of the four acceptors tested.

To further demonstrate that enzymatic activity is directly associated with this protein, the putative catalytic domain of the predicted polypeptide (amino acids 43 to 374 of SEQ ID NO: 11) was fused to a secreted form of the IgG binding domain of *Staphylococcus aureus* protein A in the mammalian expression vector pPROTA, to generate the vector pPROTA-Fuc-TV<sub>c</sub>. Since this fusion protein would lack the putative transmembrane anchoring segment of the fucosyltransferase, the

inventor expected it would be synthesized as a secreted molecule that could be affinity-purified on an IgG-containing matrix and subsequently tested for  $\alpha(1,3)$ fucosyltransferase activity. COS-1 cells transfected with the control vector pCDM7 or pPROTA generated no detectable cell-associated or released enzyme activity. However, conditioned media prepared from COS-1 cells transfected with pPROTA-Fuc-TV<sub>c</sub> contained significant quantities of  $\alpha(1,3)$ fucosyltransferase activity when assayed with N-acetyllactosmine. Virtually 100% of the released activity generated by pPROTA-Fuc-TV<sub>c</sub> is specifically retained by the IgG-Sepharose matrix. These results indicate that the protein encoded by this cloned DNA segment encodes a fucosyltransferase, and demonstrate that information sufficient to generate  $\alpha(1,3)$ fucosyltransferase activity resides within the enzyme's COOH-terminal 332 amino acids.

Taken together with the results of the flow cytometry analyses and DNA sequence analysis, these experiments indicate that plasmid pCDNA1-Fuc-TV encodes a novel  $\alpha(1,3)$ fucosyltransferase.

Experimental Procedures for Example V, "Isolation of a GDP-Fuc- $\beta$ -D-Gal(1,4)-D-GlcNAc  $\alpha(1,3)$ Fucosyltransferase (Fuc-TV) through cross-hybridization:

Cell culture. The source and growth conditions of COS-1 cells used are as previously described. See Ernst et al.; J.



Biol. Chem. (1989) 265:3436-3447 and Rajan et al, J. Biol. Chem. (1989) 264:11158-11167.

Antibodies. The anti-Lex antibody anti-SSEA-1 (mouse monoclonal IgM as ascites) was used. Solter et al, Proc. Nat. Acad. Sci. (USA) (1978), 75:5565-5569. Anti-H and anti-Lewis a antibodies (mouse monoclonal IgM, antigen affinity purified) were purchased from Chembiomed Ltd. (Edmonton, Alberta). Anti-sialyl Lewis x antibody CSLEX1 (Fukushima et al, Cancer Res. (1984) 44:5279-5285) (mouse monoclonal IgM, HPLC purified) and anti-sialyl Lewis a antibody CSLEA1 (Chia et al, Cancer Res. (1985) 45:435-437) (mouse monoclonal IgG3, ammonium sulfate precipitate) were used. Fluorescein-conjugated goat anti-mouse IgM or IgG antibodies were purchased from Sigma.

Human genomic library construction. High molecular weight human genomic DNA was prepared from peripheral blood leukocytes. Genomic DNA was subjected to partial digestion with the restriction endonuclease Sau3A. The partially digested genomic DNA was size fractionated by ultracentrifugation through a sodium chloride gradient. Fractions enriched for DNA fragments between 8 Kb and 20 Kb were ligated to XhoI digested lambda FIX (Stratagene) phage arms that had been partially filled in with dTTP and dCTP to make the ends compatible with the Sau3A fragments.

The ligation mixture was packaged in vitro with commercial packaging extracts (Stratagene), tittered on *E.*

*coli* host TAP90. Approximately  $1.0 \times 10^6$  recombinant lambda phage were screened by plaque hybridization. Plaque lifts were prepared using nitrocellulose filters (Schleicher and Schuell) and were prehybridized at 42°C for 16 hours in 50% formamide, 5X SSC, 10X Denhart's solution, and 0.1% SDS. Filters were hybridized for 72 hours at 35°C in prehybridization solution containing 10% dextran sulfate, and 100 micrograms per ml denatured salmon sperm DNA. The probe consisted of a 1.7 Kb XhoI-XbaI fragment isolated from the 5' end of a cDNA insert encoding the Lewis blood group  $\alpha(1,3/1,4)$  fucosyltransferase, which was labeled with [ $\alpha$ - $^{32}$ P] dCTP. The filters were rinsed three times for 20 minutes each at room temperature in 2X SSC and then once for 40 minutes at 50°C and 1X SSC, 0.5% SDS. Filters were then subjected to autoradiography. Eighteen independent hybridization-positive plaques were identified after 2 additional cycles of plaque hybridization. Phage DNAs were prepared from liquid lysates and were subsequently characterized by restriction endonuclease digestions and Southern blot analyses.

DNA sequence analysis. Phage DNA was digested with various restriction enzymes, and fragments homologous to the human  $\alpha(1,3/1,4)$  fucosyltransferase cDNA were gel purified and ligated into the multicloning site of pTZ18R. Representative subclones were sequenced by the dideoxy chain determination method using T7 DNA polymerase (Pharmacia LKB Biotechnology, Inc.) and oligonucleotides synthesized according to flanking

plasmid sequences and subsequently according to the insert sequence. This sequence data was used to generate additional synthetic deoxynucleotides which were then used to sequence remaining portions of the inserts. Sequence analysis was performed using the sequence analysis software package of the University of Wisconsin Genetics Computer Group.

Construction of plasmid pCDNA1-Fuc-TV

A 1.94 kb Earl-XbaI fragment was isolated from a representative phage taken from a strongly hybridizing class of phages, made blunt with the Klenow fragment of *E. coli* DNA polymerase I, and cloned into the EcoRV and XbaI sites in the mammalian expression plasmid pCDNA1 (Invitrogen). One plasmid with a single insert in the sense orientation with respect to the plasmid's CMV promoter enhancer sequences was designated pCDNA1-Fuc-TV.

FACS analysis. COS-1 cells transfected with plasmid DNAs were harvested 48-72 hours after transfection, and stained with monoclonal antibodies diluted in staining media. Anti-Lewis a and anti-H antibodies (mouse IgM monoclonal; antigen-affinity purified; Chembiomed, Edmonton) were used at 10 µg/ml. Anti-SSEA-1 (mouse monoclonal IgM; ascites) was used at a dilution of 1:1000. Anti-sialyl-Lewis x (mouse monoclonal IgM; HPLC purified from ascites) was used at 10 µg/ml. Anti-sialyl Lewis a (mouse monoclonal IgG3; ammonium sulfate precipitate of ascites) was used at a dilution of 1:500. Cells were then stained with fluorescein

isothiocyanate-conjugated goat anti-mouse IgM or IgG, as appropriate, and were then subjected to analysis on a FACScan (Becton-Dickinson).

Fucosyltransferase assays. Cell extracts containing 1% Triton X-100 were prepared from transfected COS-1 cells. Fucosyltransferase assays were performed in a total volume of 20  $\mu$ l, and contained 25 mM sodium cacodylate, pH6.2, 5 mM ATP, 10 mM L-fucose, 10 mM  $\text{MnCl}_2$ , 3  $\mu$ M GDP- $^{14}\text{C}$ -fucose, and 5  $\mu$ l of cell extract. Acceptor substrates were added to a final concentration of 20 mM. Reactions were incubated at 37°C for 1 hour and terminated by addition of 20  $\mu$ l ethanol, followed by addition of 600  $\mu$ l of distilled water. An aliquot of each reaction (50  $\mu$ l) was subjected to scintillation counting to determine total radioactivity in the reaction. Another aliquot (200  $\mu$ l) was applied to a column containing 400  $\mu$ l of Dowex 1X2-400, formate form. The flow through fraction, and 2  $\mu$ l of a subsequent water elution, were collected and pooled, and an aliquot was subjected to scintillation counting to quantitate incorporation of radioactive fucose into neutral product.

Example VI. Cloning and expression of a DNA sequence encoding a GDP-Fuc: $\beta$ -D-Gal(1,4)-D-GlcNAc  $\alpha$ (1,3)fucosyltransferase" (Fuc-TVI;DNA SEQ ID NO:13, protein SEQ ID NO:14) through cross hybridization.

Biochemical and genetic studies indicate that the human genome encodes two or more distinct GDP-L-fucose: $\beta$ -D-Galactoside 3- $\alpha$ -L-Fucosyltransferases (Potvin et al, J. Biol. Chem., 265:1615-1622, 1990; Watkins, Adv. Hum. Genet., 10:1-116, 1980). the inventor has recently described a cloned cDNA that encodes one of these enzymes, that is thought to represent the product of the human Lewis blood group locus (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990) (DNA SEQ ID NO:1 and Protein SEQ ID NO:2). In consideration of the possibility that these GDP-L-fucose: $\beta$ -D-Galactoside 3- $\alpha$ -L-Fucosyltransferases might be encoded by a family of structurally-related genes, the inventor sought to isolate other such members by cross-hybridization methods, using the cloned Lewis fucosyltransferase cDNA.

Molecular cloning of a human genomic DNA segment that crosshybridizes to the Lewis blood group  $\alpha(1,3/1,4)$ fucosyltransferase cDNA - As noted above in Examples IV and V, low stringency Southern blot hybridization experiments indicate that the coding region of the Lewis fucosyltransferase cDNA detects strongly hybridizing restriction fragments, as well as several weakly hybridizing fragments. To further examine the molecular nature of these sequences, the inventor screened a human lambda phage genomic DNA library at low stringency with the Lewis cDNA probe. A total of 18 phages were isolated from phages representing

approximately five human genomic equivalents. Southern blot analysis of 16 of these phages allowed them to be placed into three groups, based upon their restriction patterns and hybridization signal intensity strengths. Several phages representing a class with strong hybridization intensities were identified. Cross-hybridizing restriction fragments isolated from one of these phages was subcloned and sequenced.

The homologous DNA restriction fragment maintains a single long open reading frame that predicts a polypeptide with strong similarity to the Lewis blood group  $\alpha(1,3/1,4)$  fucosyltransferase cDNA - DNA sequence analysis of the cross-hybridizing subcloned fragment (SEQ ID NO:13) identified a single long open reading frame, beginning at base pair 1 and ending at base pair 1080 (Figure 7). This reading frame begins with a methionine codon that is found within a sequence context consistent with Kozak's consensus rules for mammalian translation initiation. A hydropathy analysis of the protein sequence predicted by this reading frame (SEQ ID NO:14) predicts a single hydrophobic segment at its NH<sub>2</sub>-terminus, suggesting that it represents a 359 amino acid protein (SEQ ID NO:14) that is predicted to maintain the type II transmembrane orientation typical of mammalian glycosyltransferases. Virtually the entire length of this reading frame shares a strikingly high amount of amino acid (not shown) and nucleic acid sequence identity with the corresponding portion of the Lewis fucosyltransferase cDNA

(Figure 7). Because of the substantial sequence similarity between these two DNA sequences, and their derived protein sequences, we expected this new cross-hybridizing sequence to represent a single exonic sequence, representing a heretofore undefined gene, that encodes a fucosyltransferase.

The homologous DNA restriction fragment determines expression of an  $\alpha(1,3)$ fucosyltransferase - To determine if this segment encodes a functional fucosyltransferase, a 1.2 kb fragment containing the entire open reading frame was generated by the polymerase chain reaction and was cloned into a mammalian expression vector, and the resulting plasmid (pCDNA1-Fuc-TVI) was introduced into mammalian host cells by transfection. COS-1 cells were used as hosts for these experiments since these cells normally express virtually undetectable  $\alpha(1,3)$ - and  $\alpha(1,4)$ fucosyltransferase activities (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990). Likewise, COS-1 cells do not normally express detectable amounts of cell surface  $\text{Gal}\beta 1\rightarrow 4[\text{Fuc}\alpha(1\rightarrow 3)]\text{GlcNAc}-(\text{Lewis x}, \text{SSEA-1})$  or  $\text{Gal}\beta 1\rightarrow 3[\text{Fuc}\alpha(1\rightarrow 4)]\text{GlcNAc}-(\text{Lewis a})$  moieties, whereas they do maintain surface-display of non-fucosylated type II and type I oligosaccharide precursors necessary for the construction of such molecules (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990). The vector pCDNA1 was used since this plasmid efficiently transcribes exogenous, subcloned sequences in COS-1 hosts by virtue of the cytomegalovirus immediate early promoter sequences in the

vector, and is maintained in these cells as a multicopy episome (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990). COS-1 cells transfected with pCDNA1-Fuc-TVI were first analyzed by flow cytometry to detect de novo, vector-dependent surface expression of these oligosaccharides. A substantial fraction of these transfected cells exhibited bright staining with a monoclonal antibody directed against the Lewis x moiety ( $\text{Gal}\beta(1\rightarrow4)[\text{Fuc}\alpha(1\rightarrow3)]\text{GlcNAc-}$ ) (Figure 8), whereas cells transfected with the pCDNA1 vector without insert did not express this determinant. By contrast, COS-1 cells transfected with pCDNA1-Fuc-TVI, or with its control plasmid, did not stain with antibodies specific for the type I-based Lewis a trisaccharide (Figure 8). Taken together, these results are consistent with the results of the DNA sequence analysis indicating that this segment encodes an  $\alpha(1,3)$  fucosyltransferase competent to utilize neutral type II oligosaccharide precursor, but that the enzyme cannot utilize type I glycoconjugates and thus does not exhibit  $\alpha(1,4)$  fucosyltransferase activity.

There is evidence supporting the possibility that one or more human  $\alpha(1,3)$  fucosyltransferases exist that can utilize type II acceptors whose terminal galactose residues are substituted with  $\alpha(2,3)$  sialic acid moieties (Potvin et al, J. Biol. Chem., 265:1615-1622, 1990; Holmes et al, J. Biol. Chem., 261:3737-3743, 1986; Palcic et al, Carbohydr. Res., 190:1-11, 1989). Such enzymes can fucosylate these molecules



to form the sialyl-Lewis x determinant (NeuAc $\alpha$ (2 $\rightarrow$ 3)Gal $\beta$ (1 $\rightarrow$ 4)[Fuc $\alpha$ (1 $\rightarrow$ 3)]GlcNAc-). The Lewis fucosyltransferase, for example, is competent to perform this reaction (Palcic et al, Carbohydr. Res., 190:1-11, 1989; Lowe et al, Cell, 63:475-484, 1990). It was therefore of interest to determine if the fucosyltransferase apparently encoded by plasmid pCDNA1- $\alpha$ (1,3)Fuc-TVI would be capable of constructing sialyl-Lewis x determinants. COS-1 cells maintain surface-expressed glycoconjugates terminating in (NeuAc $\alpha$ (2,3)Gal $\beta$ (1,4)GlcNAc- (Lowe et al, Cell, 63:475-484, 1990; Fukuda et al, J. Biol. Chem., 263:5314-5318, 1988); these represent acceptor substrates for sialyl-Lewis x construction determined via the action of enzymes encoded by transfected fucosyltransferase expression vectors (Lowe et al, Cell, 63:475-484, 1990). COS-1 cells were therefore transfected with plasmid pCDNA1-Fuc-TVI, stained with a monoclonal anti-sialyl Lewis x antibody, and subjected to flow cytometry analysis. A significant amount of staining was detected, relative to control cell transfected with the vector alone, or relative to pCDNA1-Fuc-TVI-transfected cells stained with a negative control antibody (anti-H, Figure 8). However, these cells did not stain with an antibody specific for the type I-based, sialyl-Lewis a determinant (NeuAc $\alpha$ (2,3)Gal $\beta$ (1,3)[Fuc $\alpha$ (1,4)]GlcNAc-) (Figure 8). By contrast, the inventor has previously demonstrated that a substantial fraction of COS-1 cells transfected with the Lewis

fucosyltransferase expression plasmid pCDM7- $\alpha(1,3/1,4)$ FT exhibit bright staining with the sialyl-Lewis x and sialyl-Lewis a antibodies (Lowe et al, Cell, 63:475-484, 1990), as predicted by biochemical analysis of the acceptor substrate specificity of this enzyme (Palcic et al, Carbohydr. Res., 190:1-11, 1989).

The conclusion that plasmid pCDNA1-Fuc-TVI encodes an  $\alpha(1,3)$ fucosyltransferase was confirmed by biochemical analysis of the acceptor substrate requirements of the enzyme in extracts of COS-1 cells transfected with plasmid pCDNA1-Fuc-TVI. As expected, the enzyme in these extracts utilized the type II disaccharide acceptor N-acetyllactosmine, to yield the predicted product  $\text{Gal}\beta(1\rightarrow4)[\text{Fu}\alpha(1\rightarrow3)]\text{GlcNAc}$ . The enzyme also efficiently utilized the trisaccharide  $\text{NeuAc}\alpha(2,3)\text{Gal}\beta(1,4)\text{GlcNAc}$  to form the sialyl Lewis X tetrasaccharide (Table 2). Under these conditions, other type II molecules, including lactose, and the  $\alpha(1,2)$ fucosylated type II acceptor  $\text{Fu}\alpha(1\rightarrow2)\text{Gal}\beta(1\rightarrow4)\text{Glc}$ , did not function as acceptor substrates for the fucosyltransferase in these extracts, with any detectable efficiency. The type I substrate lacto-N-biose I was also not utilized by this enzyme. This suggests that the enzyme can function effectively only as an  $\alpha(1,3)$ fucosyltransferase, as also suggested by the flow cytometry analyses. The acceptor preferences exhibited by this enzyme contrast significantly with those exhibited by the Lewis fucosyltransferase, which is

able to efficiently utilize each of the four acceptors tested (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990; Mollicone et al, Eur. J. Biochem., 191:169-176, 1990). Taken together with the results of the flow cytometry analyses presented in Figure 8, and DNA sequence analysis, these biochemical experiments, summarized in Table 2, indicate that plasmid pCDNA1-Fuc-TVI encodes a novel fucosyltransferase with its own distinct acceptor specificity.

Since the protein sequence of Fuc-TVI is so very similar to the sequences of FUC-TV and FUC-TIII, it may be expected that a catalytically active, secreted protein A-Fuc-TVI fusion protein may be generated by fusing residues 43 through 359 of Fuc-TVI (SEQ ID NO:14) to the protein A segment, in a manner identical to that used to generate pPROTA- $\alpha$ (1,3/1,4)FT<sub>c</sub>, and pPROTA-Fuc-TV<sub>c</sub>.

Experimental Procedures for Example VI "Cloning and expression of a DNA sequence encoding GDP-Fuc- $\beta$ -D-Gal(1,4)-D-GlcNAc  $\alpha$ (1,3) fucosyltransferase" (Fuc-TVI; DNA SEQ ID NO:<sup>13</sup>14, protein SEQ ID NO:<sup>14</sup>15) through cross hybridation.

Cell culture. The source and growth conditions of COS-1 cells are as previously described (Ernst et al, J. Biol. Chem., 265:3436-3447, 1989; Rajan et al, J. Biol. Chem., 264:11158-11167, 1989).

Antibodies. The anti-Lex antibody anti-SSEA-1 (Solter et al, Proc. Natl. Acad. Sci. USA, 75:5565-5569, 1978) (mouse monoclonal IgM as ascites) was provided by Dr. Davor Solter (Wistar Institute, Philadelphia). Anti-H and anti-Lewis a antibodies (mouse monoclonal IgM, antigen affinity purified) were purchased from Chembiomed Ltd. (Edmonton, Alberta). Anti-sialyl Lewis x antibody CSLEX1 (Fukushima et al, Cancer Res., 44:5279-5285, 1984) (mouse monoclonal IgM, HPLC purified) and anti-sialyl Lewis a antibody CSLEA1 (Galton et al, Ninth Int. Convoc. Immuno., Amherst, NY, pp. 117-125, Karger, Basel; Chia et al, Cancer Res., 45:435-437, 1985) (mouse monoclonal IgG3, ammonium sulfate precipitate) were provided by Dr. P. Terasaki (UCLA, Los Angeles). A pooled mouse IgG antibody preparation (MsIg) was purchased from Coulter. Fluorescein-conjugated goat anti-mouse IgM or IgG antibodies were purchased from Sigma.

Human genomic library construction. High molecular weight human genomic DNA was prepared from peripheral blood leukocytes as described previously (Ernst et al, J. Biol. Chem., 265:3436-3447, 1989). Genomic DNA was subjected to partial digestion with the restriction endonuclease Sau3A. The partially digested genomic DNA was size fractionated by ultracentrifugation through a sodium chloride gradient. Fractions enriched for DNA fragments between 8 Kb and 20 Kb were ligated to XhoI digested lambda FIX (Stratagene) phage

arms that had been partially filled in with dTTP and dCTP to make the ends compatible with the *Sau*3A fragments. The ligation mixture was packaged in vitro with commercial packaging extracts (Stratagene), titered on *E. coli* host TAP90 (Patterson et al, Nucl. Acids. Res., 15:6298, 1987).

Approximately  $1.0 \times 10^6$  recombinant lambda phage were screened by plaque hybridization. Plaque lifts were prepared using nitrocellulose filters (Schleicher and Schuell) and were prehybridized at 42°C for 16 hours in 50% formamide, 5X SSC, 10X Denhart's solution, and 0.1% SDS. Filters were hybridized for 72 hours at 35°C in prehybridization solution containing 10% dextran sulfate, and 100 micrograms per ml denatured salmon sperm DNA. The probe consisted of a 1.7 Kb *Xho*I-*Xba*I fragment isolated from the 5' end of a cDNA insert encoding the Lewis blood group  $\alpha(1,3/1,4)$  fucosyltransferase (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990), which was labeled (Feinberg et al, Anal. Biochem., 132:6-13, 1983) with [ $\alpha$ - $^{32}$ P] dCTP. The filters were rinsed three times for 20 minutes each at room temperature in 2X SSC and then once for 40 minutes at 50°C and 1X SSC, 0.5% SDS. Filters were then subjected to autoradiography. Eighteen independent hybridization-positive plaques were identified after 2 additional cycles of plaque hybridization. Phage DNAs were prepared from liquid lysates (Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Lab., Cold Spring Harbor, NY, 1982) and were subsequently characterized

by restriction endonuclease digestions and Southern blot analyses.

DNA sequence analysis. Phage DNA was digested with various restriction enzymes, and fragments homologous to the human  $\alpha(1,3/1,4)$ fucosyltransferase cDNA were gel purified and ligated into the multicloning site of pTZ18. Representative subclones were sequenced by the dideoxy chain determination method (Sanger et al, Proc. Natl. Acad. Sci. USA, 74:5463-5467, 1977) using T7 DNA polymerase (Pharmacia LKB Biotechnology, Inc.) and oligonucleotides synthesized according to flanking plasmid sequences and subsequently according to the insert sequence. This sequence data was used to generate additional synthetic deoxynucleotides which were then used to sequence remaining portions of the inserts. Sequence analysis was performed using the sequence analysis software package of the University of Wisconsin Genetics Computer Group (Devereux et al, Nucl. Acids. Res., 12:387-395, 1984).

Transfection and expression of the insert in pCDNA1-Fuc-TVI.

A 1.2 kb fragment was generated by the PCR, using DNA isolated from a representative phage taken from a strongly hybridizing class of phages, and cloned into the HindIII site in the mammalian expression plasmid pCDNA1 (Invitrogen). One plasmid with a single insert in the sense orientation with respect to

the plasmid's CMV promoter enhancer sequences was designated pCDNA1-Fuc-TVI.

FACS analysis. COS-1 cells transfected with plasmid DNAs were harvested (Rajan et al, J. Biol. Chem., 264:11158-11167, 1989) 48-72 hours after transfection, and stained with monoclonal antibodies diluted in staining media, as previously described (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990; Ernst et al, J. Biol. Chem., 265:3436-3447, 1989). Anti-Lewis a and anti-H antibodies (mouse IgM monoclonal; antigen-affinity purified; Chembiomed, Edmonton) were used at 10 µg/ml. Anti-SSEA-1 (mouse monoclonal IgM; ascites) was used at a dilution of 1:1000. Anti-sialyl-Lewis x (mouse monoclonal IgM; HPLC purified from ascites) was used at 10 µg/ml. Anti-sialyl Lewis a (mouse monoclonal IgG3; ammonium sulfate precipitate of ascites) was used at a dilution of 1:1000. Control mouse IgG3 antibody (MsIg, Coulter) was used at a concentration of 10 µg/ml. Anti-VIM-2 antibody (mouse monoclonal IgM; ascites) was used at a dilution of 1:200. Cells were then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgM or IgG, as appropriate, and were then subjected to analysis on a FACScan (Becton-Dickinson), as described previously (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990).

Fucosyltransferase assays. Cell extracts containing 1% Triton X-100 were prepared from transfected COS-1 cells, using procedures described previously (Kukowska-Latallo et al, Genes Devel., 4:1288-1303, 1990). Fucosyltransferase assays were performed in a total volume of 20  $\mu$ l, and contained 50 mM sodium cacodylate, pH 6.2, 5 mM ATP, 10 mM fucose, 20 mM  $\text{MnCl}_2$ , 3  $\mu$ M GDP- $^{14}\text{C}$ -fucose, and 5  $\mu$ l (30  $\mu$ g protein) of cell extract. Acceptor substrates were added to a final concentration of 20 mM. Reactions were incubated at 37°C for 1 hour and terminated by addition of 20  $\mu$ l ethanol, followed by addition of 600  $\mu$ l of distilled water. An aliquot of each reaction (50  $\mu$ l) was subjected to scintillation counting to determine total radioactivity in the reaction. Another aliquot (200  $\mu$ l) was applied to a column containing 400  $\mu$ l of Dowex 1X2-400, formate form (Rajan et al, J. Biol. Chem., 264:11158-11167, 1989). The flow through fraction, and 2  $\mu$ l of a subsequent water elution, were collected and pooled, and an aliquot was subjected to scintillation counting to quantitate incorporation of radioactive fucose into neutral product.



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TABLE 2

Substrate Utilization Properties of Human  $\alpha(1,3)$ Fucosyltransferases

Acceptor substrate	Product Name	Relative Activity (%) with each $\alpha(1,3)$ Fucosyltransferase			
		Fuc-TIII	Fuc-TIV	Fuc-TV	Fuc-TVI
N-acetyllactosamine (20 mM)	Lewis x	100	100	100	100
lactose (20 mM)	Lewis x	145	3	11	<1
$\alpha(2,3)$ sialyllactosamine (20 mM)	sialyl Lewis x	56	<1	115	110
2'-fucosyllactose (5 mM)	Lewis y	254	6	42	<1
lacto-N-biose I (20 mM)	Lewis a	420	<1	10	<1

Table 2 presents the relative product formation rates obtained with low molecular weight acceptor substrates using cell extracts containing recombinant human  $\alpha(1,3)$ fucosyltransferases expressed in transfected COS-1 cells, as described in the preceding sections. Fucosyltransferase assays were performed as described in detail in Lowe et al, J. Biol. Chem., (1991), 266:17467-17477; Weston et al, J. Biol. Chem. (1992), 267:4152-4160; and Kukowska-Latallo et al, Genes. Devel., (1990), 4:1288-1303. For each enzyme, the same extract was used, with saturating amounts of each acceptor oligosaccharide (20 mM, except for 2'-fucosylactose which was used at 5 mM), and GDP-[ $^{14}$ C]fucose was present at 3  $\mu$ M. Reaction times and enzyme amounts were adjusted to ensure a linear rate of product formation (less than 15% of the GDP-fucose substrate consumed). Products were separated by column chromatography and quantitated by liquid scintillation counting, and their structures were confirmed by high performance liquid chromatography, as described in Lowe et al, J. Biol. Chem., (1991), 266:17467-17477; Weston et al, J. Biol. Chem. (1992), 267:4152-4160; and Kukowska-Latallo et al, Genes. Devel., (1990), 4:1288-1303.

#### Example VII. Chimeric Enzymes.

The abbreviations used are Lewis a ( $Le^a$ ), Gal $\beta$ 1-3[Fuc $\alpha$ 1-4]GlcNAc; Lewis x ( $Le^x$ ), Gal $\beta$ 1-4[Fuc $\alpha$ 1-3]GlcNAc; sialyl Lewis x ( $sLe^x$ ), NeuNAc $\alpha$ 2-3Gal $\beta$ 1-4[Fuc $\alpha$ 1-3]GlcNAc; sialyl Lewis a ( $sLe^a$ ), NeuNAc $\alpha$ 2-3Gal $\beta$ 1-3[Fuc $\alpha$ 1-4]GlcNAc; H,

Fuc $\alpha$ 1-2Gal $\beta$ 1-4GlcNAc  $\alpha$ (1,3)fucosyltransferase;  $\alpha$ (1,3)Fuc-T, GDP-fucose: $\beta$ -D-N-acetylglucosaminide 3- $\alpha$ -L-fucosyltransferase; PCR, polymerase chain reaction; kb, kilobase; bp, base pair.

Nomenclature - Previous literature (Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584; Natsuka, S., and Lowe, J.B. (1994) *Curr. Opin. Struct. Biol.*, vol. 4, 683-691) has summarized the properties of, and terminology for, Fuc-TIII (the human Lewis blood group  $\alpha$ (1,3/1,4)fucosyltransferase; Lowe, J.B., et al (1990) *Cell*, vol. 63, 475-484; Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303), Fuc-TV (Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160; Koszdin, K.L., and Bowen, B.R. (1992) *Biochem. Biophys. Res. Commun.*, vol. 187, 152-157), and Fuc-TVI (the "plasma-type"  $\alpha$ (1,3)fucosyltransferase; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584; Mollicone, R., et al (1994) *J. Biol. Chem.*, vol. 269, 12662-12671).

Site-directed mutagenesis - Site directed mutagenesis procedures were used to install novel restriction sites in the wild type Fuc-TIII and Fuc-TVI coding sequences to facilitate excision and replacement of DNA segments corresponding to the various Fuc-TIII and Fuc-TVI. DNA fragments corresponding to the coding regions of Fuc-TIII and Fuc-TVI were first generated by the polymerase chain reaction (Saiki, R.K., et al (1985) *Science*, vol. 230, 1370-1374). Fifty nanograms of plasmid template (pCDM7- $\alpha$ (1,3/1,4)FT, (Kukowska-Latallo, J.F.,

et al (1990) *Genes Devel.*, vol. 4, 1288-1303), or pcDNAI-Fuc-TVI, (Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584) were used in reactions with the Gene Amp Kit (Perkin, Elmer, Cetus). Primers were used that flank the coding region of each gene (Fuc-TIII sense primer is GCGCGAATTCATGGATCCCCTGGGTGCAGCCAAGCCACAAT<sup>(SEQ ID NO 18)</sup>, Fuc-TIII anti-sense primer is GCGCTCTAGAGGCAGATGAGGTTCCCGGCAGCCCAGGCAC<sup>(SEQ ID NO 19)</sup>; Fuc-TVI: sense primer is GCGCGAATTCTCCTCTCTCCCCACTTCCCAGAGACT<sup>(SEQ ID NO 20)</sup>, Fuc-TVI anti-sense primer is CGCGTCTAGAGGTGAAGCTTCAGGCAAACGAGTCCTTAGGT<sup>(SEQ ID NO 21)</sup>). Primers were designed to yield PCR products with an *EcoRI* site at the 5' end and an *XbaI* site at the 3' end. Twenty cycles of amplification were performed, consisting of 1.5 min at 94°C, followed by annealing and extension for 3.5 min at 72°C. The PCR products were digested with *EcoRI* and *XbaI* and were cloned between these sites in the vector pcDNAI (Invitrogen), using standard molecular biology procedures (Maniatis, T., et al (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, NY)). The DNA sequence of the insert in each vector was determined using the dideoxy chain termination method (Sanger, F., et al (1977) *Proc. Natl. Acad. Sci. USA*, vol. 74, 5463-5467) and T7 DNA polymerase (Sequenase, US. Biochemical Corp.). A representative error-free clone was chosen for each gene (termed pcDNAI-Fuc-TIII and pcDNAI-Fuc-TVI) and was used for expression and mutagenesis procedures.

The coding regions of Fuc-TIII and Fuc-TVI were mutated to create unique inter-subdomain restriction endonuclease sites. Restriction sites were chosen to correspond to positions within the conserved amino acid sequence between subdomains, to maintain the wild type protein sequence, and to be unique within the plasmid vector. For Fuc-TIII, this involved installation of five new inter-subdomain restriction sites, and elimination of an *MscI* site. Creation of six new inter-subdomain restriction sites was required for Fuc-TVI. Mutagenesis was completed using the p-Alter Mutagenesis Kit (Promega), after subcloning the inserts in pCDNAI-Fuc-TIII and pCDNAI-Fuc-TVI between the *EcoRI* and *XbaI* sites of the plasmid p-Alter (Promega). Simultaneous multiple oligonucleotide site directed mutagenesis was performed on single strand templates, using procedures recommended by the manufacturer. Sequences of the mutagenic oligonucleotides are shown in Figure 9b. Candidate mutant clones were screened for the desired restriction sites by restriction enzyme digestion. Mutated clones containing each of the desired mutations were sequenced (Sanger, F., et al (1977) *Proc. Natl. Acad. Sci. USA*, vol. 74, 5463-5467) in their entirety to identify ones that contained no undesired additional mutations. Single, appropriately mutated but fully correct clones for p-Alter-Fuc-TIII and p-Alter-Fuc-TVI, were digested with *EcoRI* and *XbaI*, and the released mutated inserts were recloned between the *EcoRI* and *XbaI* sites of pCDNAI.

Construction of recombinant Fuc-T Chimeras via cassette mutagenesis - All recombinant  $\alpha(1,3)$ Fuc-T chimeras except those involving subdomain 3 exchange (Fuc-TC7 and Fuc-TC13) were constructed by exchanging various restriction fragments, flanked by unique restrictions sites, corresponding to the subdomains that differ between Fuc-TIIII and Fuc-TVI. In chimeras constructed via subdomain 3 exchange (Fuc-TC7 and Fuc-TC13, Fig. 11), Fuc-TIIII and Fuc-TVI sense and anti-sense oligonucleotide primers encoding subdomain 3 were annealed, phosphorylated (Maniatis, T., et al (1989) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, NY)), and the resulting 39 bp "synthetic" double stranded DNA segments were cloned between the *Sma*I and *Bst*1071 sites in pCDNAI-Fuc-TIIII or pCDNAI-Fuc-TVI. The insert in each chimera was sequenced in its entirety.

Finally, chimera Fuc-TC21 was constructed by replacing subdomains 4 and 5 of Fuc-TIIII with a region corresponding to subdomains 4 and 5 of Fuc-TV. The PCR, and oligonucleotide primers containing a 5' *Bst* 11071 site and a 3' *Bgl* II site (sense primer: CCGACTCCAGTGTATACCCACAGGCAGAC; anti-sense primer: GTCGGAGTCAGATCTGTAGGACAT), were used to generate this segment. PCR amplification conditions were chosen to minimize Taq polymerase-induced errors (annealing at 94°C for 3 min; 20 cycles consisting of 1 min at 94°C, 45 sec at 65°C, and 1 min at 72°C; a final 7 min extension at 72°C). The PCR product was digested with *Bst* 11071 and *Bgl* II, and the resulting fragment was used to replace the corresponding *Bst* 11071 and

Bgl II fragment (subdomains 4 and 5) of the Fuc-TIIII coding region. An error free clone identified by sequence analysis was termed Fuc-TC21, and was used for transfection analyses.

Transfection of COS-7 cells - COS-7 cells were cultured and transfected (DEAE-dextran method) as previously described (Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303; Natsuka, S., et al (1994) *J. Biol. Chem.*, vol. 269, 16789-16794). Cells were transfected with 20 µg of negative control vector plasmid DNA (pcDNAI), or with the plasmid DNAs pcDNAI-Fuc-TIIII, pcDNAI-Fuc-TVI, or each of the various pcDNAI-Fuc-TCs. Cells were also co-transfected with 1 µg of the plasmid pCDM8-CAT (Smith, P.L., and Lowe, J.B. (1994) *J. Biol. Chem.*, vol. 269, 15162-15171) to allow for normalization of flow cytometry and Western blot data to transfection efficiency.

Antibodies - Anti-Lewis x (anti-SSEA-1, IgM, as ascites; Solter, D., and Knowles, B.B. (1978) *Proc. Natl. Acad. Sci. USA*, vol. 75, 5565-5569) was purchased from the Developmental Studies Hybridoma Cell Bank (Iowa City, Iowa)). Anti-sialyl Lewis x (CSLEX1, IgM; Fukushima, K., et al (1984) *Cancer Res.*, vol. 44, 5279-5285) and anti-sialyl-Lewis a (CSLEA1, IgG; Chia, D., et al (1985) *Cancer Res.*, vol. 45, 435-437) monoclonal antibodies were purchased from the UCLA Tissue Typing Laboratory (Los Angeles, CA). Anti-Lewis a ascites and anti-H ascites were produced from hybridoma cell lines 151-6-A7-9 (Blaszczak, M., et al (1984) *Arch. Biochem. Biophys.*, vol. 233, 161-168) and BE2 (Young, W.W., et al (1981) *J. Biol.*

*Chem.*, vol. 256, 10967-10972) obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Flow cytometry analysis - Transfected COS-7 cells were harvested 72 h after transfection, and were stained with antibodies diluted in staining media (Ernst, L.K., et al (1989) *J. Biol. Chem.*, vol. 265, 3436-3447), using procedures previously described (Lowe, J.B., et al (1990) *Cell*, vol. 63, 475-484; Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303; Macher, B.A., et al (1991) *Glycobiology*, vol. 1, 577-584). Anti-Lewis a and anti-H ascites were used at a dilution of 1:500 and 1:100 respectively. Anti-SSEA-1 ascites was used at a dilution of 1:1000. Anti-sialyl Lewis a was used at a concentration of 10.8 µg/ml. Anti-sialyl Lewis x was used at a dilution of 10 µg/ml. Cells were subsequently washed, and then stained with either a fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Vector) used at a dilution of 1:200, or with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma), used at a dilution of 40 µg/ml. Stained cells were then analyzed on a Becton-Dickinson FACScan (Lowe, J.B., et al (1990) *Cell*, vol. 63, 475-484; Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303; Macher, B.A., et al (1991) *Glycobiology*, vol. 1, 577-584). Thresholds for antigen-positivity were set at a fluorescence intensity level that excludes 99% of COS-7 cells that had been transfected with the negative control vector and stained with the test antibody. These thresholds were set individually for each test antibody, on the day of



each experiment, before the experimental transfectants were analyzed.

Fucosyltransferase assays - Extracts for  $\alpha(1,3)$ Fuc-T assays were prepared by harvesting COS-7 cells 72 hours after transfection, and solubilizing the cell pellets in 1% Triton X-100 (Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303; Lowe, J.B., et al (1991) *J. Biol. Chem.*, vol. 266, 17467-17477; Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160; Weston, B. W., et al (1992) *J. Biol. Chem.* vol. 267, 24575-24584). Cell extract protein contents were determined with a micro BCA protein assay reagent (Pierce). Fucosyltransferase assays were performed in a final volume of 20  $\mu$ l, and contained 25 mM sodium cacodylate (pH 6.2), 5 mM ATP, 10 mM L-fucose, 20 mM  $MnCl_2$  and 3 mM GDP- $[^{14}C]$ fucose. Cell extract protein concentration was varied to approximate linear reaction rate conditions (net  $[^{14}C]$ -fucose incorporation rate of a less than twenty percent of total input GDP- $[^{14}C]$ fucose radioactivity). Concentrations of low molecular weight acceptor substrates (GalB1 $\rightarrow$ 4GlcNAc, N-acetyllactosamine, LacNAc; or GalB1 $\rightarrow$ 3GlcNAc, lacto-N-biose I, LNB-I) were varied between 0.25 mM and 167 mM, for apparent  $K_m$  determinations. Reactions were incubated at 37°C for one hour and were terminated by the addition of 20  $\mu$ l ethanol, followed by dilution with 560 ml water. The total radioactivity in each reaction was determined by subjecting an aliquot to scintillation counting. To assess the amount of radioactive fucose incorporated into product, an additional

aliquot was applied to a Dowex 1x2-400 column in the formate form (Sasaki, K., et al (1994) *J. Biol. Chem.*, vol. 269, 14730-14737; Natsuka, S., et al (1994) *J. Biol. Chem.*, vol. 269, 16789-16794; Macher, B.A., et al (1991) *Glycobiology*, vol. 1, 577-584; Natsuka, S., and Lowe, J.B. (1994) *Curr. Opin. Struct. Biol.*, vol. 4, 683-691). The flow through fraction plus an additional 2 ml of a water column "wash" was collected, pooled and subjected to scintillation counting. Control assays containing no added acceptor substrate were also completed in parallel. Radioactivity in the flow through fraction, obtained in the absence of added acceptor, was no more than 1 % of the total added radiolabel.

In some instances, a fucosyltransferase was found to maintain a very low inherent specific activity towards one or both low molecular weight acceptor substrates, such that the rate of transfer was found to be directly proportional to acceptor substrate concentrations between 0.25 mM and at least 167 mM. In these instances, the data in Table 3 indicate that the apparent Michaelis-Menten constant exceeds 50 mM. As a measure of the relative efficiencies with which the enzymes utilize *N*-acetyllactosamine and lacto-*N*-biose I, the specific activity of each enzyme was determined, for each of these two acceptor substrates (20 mM concentration). The ratio of the specific activity was calculated and is displayed in Table 3.

Chloramphenicol acetyltransferase assays - Transfected COS-7 cells were harvested 72 h after transfection, and extracts were prepared from them by sonicating the cells in

0.25M Tris-HCl (pH 8.0). Chloramphenicol acetyltransferase assays were performed as previously described (Smith, P.L., and Lowe, J.B. (1994) *J. Biol. Chem.*, vol. 269, 15162-15171; Ausubel, J., and Frederick, M. (1987) *Molecular Biology-Laboratory Manuals*, Vols. 1 and 2, John Wiley and Sons, New York). Total cell extract protein in each reaction was adjusted to produce less than 15% incorporation of [ $^{14}$ C]-chloramphenicol during a 1 h incubation at 37°C.

Western Blot analysis - Cell extracts were prepared from transfected COS-7 cells 72 h after transfection. Extracts contained 50 mM Tris-HCl (pH 6.8), 10% SDS, and 10% glycerol. Extracts were boiled for 3 min immediately after preparation, and were stored frozen until use. Protein content was determined using the BCA reagent procedure. Extracts were prepared for SDS-PAGE by adding dithiothreitol to a final concentration of 0.1 M, and bromophenol blue to a final concentration of 0.05%. Samples were then boiled and fractionated by electrophoresis through a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to a PVDF membrane (Biorad). The membrane was rinsed and then blocked for 12-14 h at 4°C in phosphate buffered saline, pH 7.4, containing 10% bovine serum albumin and 0.2% Tween 20. The blot was washed at room temperature in phosphate buffered saline, pH 7.4, 0.2% Tween 20 and was probed with a 1:1000 dilution of a polyclonal rabbit antiserum prepared against a fragment of the human Fuc-TIIII catalytic domain expressed in *E. coli*. Alternatively, an identically

prepared blot was probed with a 1:1000 dilution of pre-immune rabbit serum. The blot was then washed, and then probed with a 1:5000 dilution of a horse radish peroxidase-conjugated anti-rabbit immunoglobulin (Sigma). The blot was then rinsed, exposed to ECL reagent (Amersham, UK), and subjected to autoradiography.

## RESULTS

Analysis of COOH-terminal subdomain exchanges - type I substrate utilization is determined by sequences in the NH<sub>2</sub>-terminal "hypervariable" segment - The amino acid sequences of human Fuc-TIII and Fuc-TVI differ from each other at only 7 corresponding positions, COOH-terminal to residue 152/151 in Fuc-TIII/Fuc-TVI (Fig 9a). By contrast, their amino acid sequences differ substantially in a "hypervariable" region bounded by their transmembrane segments (residue 37) and the beginning of their "sequence-constant" COOH-terminal domains (residues 152 and 151). It therefore seemed most likely that peptide sequence(s) within their respective "hypervariable" segments determine whether or not each enzyme can (Fuc-TIII; Lowe, J.B., et al (1990) *Cell*, vol. 63, 475-484; Kukowska-Latallo, J.P., et al (1990) *Genes Devel.*, vol. 4, 1288-1303) or cannot (Fuc-TVI; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584) (Fig 9a) efficiently utilize type I acceptor substrates.

This hypothesis was tested by first characterizing the substrate utilization patterns of recombinant Fuc-T chimeras

in which the nearly identical COOH-termini of Fuc-TIIII and Fuc-TVI were exchanged with each other (Figs. 9a and 10). Substrate utilization was determined by expressing recombinant chimeras in COS-7 cells, which maintain acceptor substrate precursors for surface-localized Le<sup>x</sup>, sLe<sup>x</sup>, Le<sup>a</sup> and sLe<sup>a</sup> epitopes (Lowe, J.B., et al (1990) *Cell*, vol. 63, 475-484; Kukowska-Latallo, J.F., et al (1990) *Genes Devel.*, vol. 4, 1288-1303), and characterizing the chimera-determined cell surface glycosylation patterns of the transfected cells using flow cytometry.

Four chimeras informative for the COOH-termini of these enzymes were evaluated. In two of these chimeras (Fuc-TC1 and Fuc-TC3), the exchanged segments were between amino acid residue 301 of Fuc-TIIII or 300 of Fuc-TVI (at positions corresponding to a BstXI site in both genes; Fig. 9a and Fig. 10) and the COOH-terminus of each enzyme. This represents a 60 amino acid long peptide segment in Fuc-TIIII, and a 59 residue long peptide in Fuc-TVI. These segments differ from each other at three amino acid positions and by the presence (Fuc-TIIII) or absence (Fuc-TVI) of a valine residue at position 353 (in Fuc-TIIII) (Fig. 9a). In the other two chimeras (Fuc-TC2 and Fuc-TC4; Fig. 10), the entire COOH-terminal regions were exchanged. These transposed segments encompass all seven amino acid sequence differences distal to the BglII site corresponding to amino acid 160 in Fuc-TIIII and 159 in Fuc-TVI (Figs. 9a and b and 10). COS-7 cells transfected with chimera Fuc-TC3 and Fuc-TC4 (which maintain

the NH<sub>2</sub>-terminal segment of Fuc-TVI, and portions of the COOH-terminus of Fuc-TIII) express the Le<sup>x</sup> and sLe<sup>x</sup> antigens, but not the type I-based Le<sup>a</sup> or sLe<sup>a</sup> epitopes (Fig. 10). By contrast, COS-7 cells transfected with chimera Fuc-TC1 and chimera Fuc-TC2 (which maintain the NH<sub>2</sub>-terminal segment of Fuc-TIII, and portions of the COOH-terminus of Fuc-TVI) express the Le<sup>x</sup>, Le<sup>a</sup> and sLe<sup>a</sup> epitopes; however, neither chimera determines expression of the sLe<sup>x</sup> antigen (Fig. 10). The reason that these latter two chimeras do not yield sLe<sup>x</sup> expression is not known. Nonetheless, these results clearly demonstrate that the NH<sub>2</sub>-terminal "hypervariable" segments of Fuc-TIII and Fuc-TVI determine their respective abilities to utilize or disregard the type I acceptor substrates expressed by COS-7 cells.

This conclusion is reinforced by *in vitro* determinations of the relative efficiencies with which the fucosyltransferases utilize a pair of representative, neutral low molecular weight type I and type II acceptor substrates, N-acetyllactosamine and lacto-N-biose I (Table 3). Wild type Fuc-TIII exhibits similar Michaelis-Menten constants for these type II and type I substrates (8.1 and 12.7 mM, respectively), although the enzyme exhibits a substantially higher relative specific activity with the type I substrate. By contrast, Fuc-TVI maintains a relatively low apparent K<sub>m</sub> for the type II acceptor (4.9 mM), a relatively high apparent Michaelis-Menten constant for the type I acceptor (greater than 50 mM), and exhibits a substantially higher relative specific activity

towards the type II substrate (Table 3). The similarity between Fuc-TC4 and Fuc-TVI observed by flow cytometry analysis (Fig. 10) is also evident from the enzymatic analyses; Fuc-TC4 exhibits apparent Michealis-Menten constants for the two acceptors, and a specific activity ratio, that do not differ substantially from those obtained with wild type Fuc-TVI (Table 3). Fuc-TC2, the chimeric counterpart to Fuc-TC4 exhibits an inherently low specific activity towards both substrates, as reflected by the high apparent Km's for the two substrates (Table 3). Nonetheless, this enzyme, like wild type Fuc-TIII, utilizes the type I acceptor substrate much more efficiently than it does the type II acceptor (Table 3). This result is also consistent with the observation that Fuc-TC2 directs type I product expression in COS-7 cells with an efficiency that equals or exceeds its ability to determine type II product expression (Fig. 10).

Analysis of single subdomain exchanges points to subdomains 4 and 5 in type I substrate utilization - To further localize peptide sequence(s) in the NH<sub>2</sub>-terminal "hypervariable" segments that are important to type I and type II acceptor utilization, we arbitrarily divided these segments into five distinct subdomains. The subdomains represent short peptide segments whose sequences are different in Fuc-TIII and Fuc-TVI. Subdomains are separated from each other by short peptide sequences that are identical in the two enzymes. We used site-directed mutagenesis procedures ("Experimental Procedures") to install restriction sites in the DNA sequences

encoding Fuc-TIIII and Fuc-TVI at positions corresponding to the inter-subdomain conserved sequence regions. These sites were located at the same relative positions in these genes, and were designed to be unique, and to conserve the wild type inter-subdomain amino acid sequences of the two enzymes (Figs. 9a and b). These sites allow the use of simple restriction fragment interchange procedures to create  $\alpha(1,3)$ Fuc-T chimeras in which one (or more) subdomain(s) from one enzyme are interchanged with the corresponding subdomain(s) in the other enzyme.

We used this approach to construct a series of seventeen additional recombinant  $\alpha(1,3)$ Fuc-T chimeras derived from Fuc-TIIII and Fuc-TVI. Each chimera was evaluated for its ability to determine expression of cell surface Le<sup>a</sup>, sLe<sup>a</sup>, Le<sup>x</sup> and sLe<sup>x</sup> epitopes in transfected COS-7 cells, using the flow cytometry procedures described above for chimeras Fuc-TC1, Fuc-TC2, Fuc-TC3, and Fuc-TC4.

A series of single subdomain transpositions were used to identify either a Fuc-TVI subdomain that inactivates Fuc-TIIII type I substrate utilization, or a Fuc-TIIII subdomain that confers type I acceptor utilization when transposed into Fuc-TVI (Figs. 11a and 11b). Each of the five Fuc-TIIII chimeras containing a single Fuc-TVI subdomain expresses each of the four cell surface antigens determined by wild type Fuc-TIIII (Fig. 11a). Nonetheless, two of these chimeras (Fuc-TC8 and Fuc-TC9), representing substitutions in subdomain 4 or 5, respectively, yield a cell surface antigen profile that



differs substantially from the Fuc-TIII profile. These two chimeras yield populations of transfectants that exhibit a relative reduction in the number of cells expressing the type I-based Le<sup>a</sup> and sLe<sup>a</sup> antigens, and a relative increase in the fraction of cells expressing the type II-based epitopes Le<sup>x</sup> and sLe<sup>x</sup>. Specifically, the ratio of the fraction of Le<sup>a</sup>-positive cells (29.8%) to the fraction of Le<sup>x</sup>-positive cells (37.2%) for wild type Fuc-TIII in this experiment is 0.80. By contrast, for Fuc-TC8, this ratio is 0.19 (9.1% Le<sup>a</sup> positive versus 47.9% Le<sup>x</sup> positive), and the ratio is 0.29 for Fuc-TC9 (10.2% Le<sup>a</sup> positive versus 35.4% Le<sup>x</sup> positive). Similar ratios are observed using results obtained with antibodies against the sialylated forms of these antigens (Fig. 11a). These two chimeras thus yield a glycosylation phenotype that is roughly intermediate between the phenotypes observed for Fuc-TIII and Fuc-TVI. In contrast to the results shown in Figure 11a, substitution of single Fuc-TIII subdomains at corresponding positions in Fuc-TVI yields a set of chimeras which each determine a glycosylation phenotype virtually identical to that determined by wild type Fuc-TVI (Fig. 11b). Taken together, these results indicate that sequences within subdomain four and five of Fuc-TIII, by themselves, can strongly, if not completely, influence the type I acceptor substrate utilization efficiency of this enzyme.

Analysis of multiple subdomain exchanges - type I  
substrate utilization is determined by composites of sequences  
in subdomains 3, 4, or 5 - To determine if peptide sequence

within two or more adjacent domains can more fully direct type I acceptor substrate discrimination, we constructed and tested a series of chimeras assembled via multiple subdomain exchanges. Chimera Fuc-TC15, containing subdomains 1, 2 and 3 from Fuc-TVI transposed into the wild type Fuc-TIII sequence, generates levels of cell surface Le<sup>x</sup>, sLe<sup>x</sup>, Le<sup>a</sup> and sLe<sup>a</sup> determinants similar to that generated by wild type Fuc-TIII (Fig. 12a). Likewise, the complementary chimera Fuc-TC17 (Fig. 12b) utilizes only type II acceptor substrates to generate Le<sup>x</sup> and sLe<sup>x</sup>, in a manner virtually identical to wild type Fuc-TVI. These results indicate that domains 1, 2, and 3, together contribute little, if anything, to type I acceptor substrate utilization.

By contrast, results obtained with an additional set of chimeras indicates that subdomains 4 and 5, when transposed together (with or without subdomain 3), strongly influence type I substrate usage. Replacement of subdomains 3, 4 and 5 in Fuc-TIII with the corresponding subdomains from Fuc-TVI yields a fucosyltransferase chimera (Fuc-TC16; Fig. 12a) that utilizes type II substrates exclusively, to generate cell surface Le<sup>x</sup> or sLe<sup>x</sup> determinants. This phenotype is virtually identical to that generated by wild type Fuc-TVI (Fig. 12a).

In a similar manner, replacement of subdomains 3, 4 and 5 in Fuc-TVI with the corresponding subdomains from Fuc-TIII creates a fucosyltransferase chimera (Fuc-TC18; Fig. 12b) with a phenotype qualitatively similar to that of wild type Fuc-TIII. Specifically, COS-7 cells expressing this chimera

express all four epitopes analyzed (Fig. 12b), although at reduced absolute levels (Le<sup>x</sup>, 8.1% positive; sLe<sup>x</sup>, 3.0% positive; Le<sup>a</sup>, 4.8% positive; and sLe<sup>a</sup>, 4.0% positive) relative to levels generated by wild type Fuc-TIIII. The diminished amount of cell surface antigen expression yielded by Fuc-TC18 is apparently not a consequence of decreased expression of the chimeric enzyme since western blot analysis of Fuc-TC18 transfectants identifies amounts of immunoreactive Fuc-TC18 in these cells that are approximately equivalent to the amounts of wild type Fuc-TVI, and nearly equivalent to wild type Fuc-TIIII accumulations. Enzyme activity assays suggest that the decreased cell surface fucosylated antigen expression we observe with this chimeric fucosyltransferase is due to the enzyme's inherently low specific activity, and not because it is mislocalized or otherwise not able to effectively interact with its intracellular target oligosaccharides.  $\alpha(1,3)$ Fuc-T activity assays indicate that the specific activity of this chimera is substantially lower than either Fuc-TIIII or Fuc-TVI, when assayed with the low molecular weight type I and type II acceptor substrates lacto-N-biose I and N-acetyllactosamine (apparent  $K_m$ 's exceeding 50 mM for these substrates; Table 3). Nonetheless, this chimeric enzyme exhibits the same *in vitro* "preference" for the type I acceptor substrate observed for wild type Fuc-TIIII (Table 3). Thus, even though the inherent efficiency of this chimeric fucosyltransferase is apparently substantially less than that of the wild type enzyme, the presence in this chimera of

subdomains 3, 4, and 5 from Fuc-TIII is associated with retention of a phenotype that is qualitatively similar to wild type Fuc-TIII, *in vitro* (Table 3) and *in vivo* (Fig. 12b).

Subdomain 3 in Fuc-TIII and Fuc-TVI varies only by the presence (Fuc-TVI) or absence (Fuc-TIII) of a consensus sequence for asparagine-linked glycosylation (Fig. 9b). This sequence, by itself, is without apparent effect on acceptor substrate utilization as judged from transfection analyses, since exchange of this subdomain alone does not alter the glycosylation phenotype determined by the corresponding mutant Fuc-TIII and Fuc-TVI chimeras (Fig. 11a, Fuc-TC7; and 11b, Fuc-TC12). Thus, when this observation is considered together with other data in Figures 10 and 11a and b, and with the results obtained with chimeras Fuc-TC15, Fuc-TC16, Fuc-TC17, and Fuc-TC18, it can be supposed that type I acceptor substrate discrimination (i.e.,  $\alpha(1,4)$ Fuc-T activity) can be conferred upon a "generic"  $\alpha(1,3)$ Fuc-T scaffold, derived from either Fuc-TIII or Fuc-TVI, by a set of sequences between the NH<sub>2</sub>-terminal boundary of subdomain 4 and the COOH-terminal boundary of subdomain 5.

This hypothesis is confirmed by analysis of chimeras in which these two subdomains have been transposed together. These chimeric  $\alpha(1,3)$ Fuc-Ts (Fuc-TC19 and Fuc-TC20) generate glycosylation phenotypes (Figs. 12a and b) that correspond nearly exactly to the phenotypes generated by chimeras constructed by exchange of subdomains 3, 4, and 5 (Figs. 12a and b). Specifically, Fuc-TC19 (subdomains 4 and 5 from Fuc-

TVI installed into Fuc-TIII) utilizes type II acceptor substrates with an efficiency approximately equivalent to wild type Fuc-TVI, but does not determine expression of type I molecules (Fig. 12a). The *in vitro* properties of this chimera are consistent with these flow cytometry results. Chimera Fuc-TC19 maintains a relatively low apparent  $K_m$  for the type II acceptor (1.1 mM), a relatively high apparent Michaelis-Menten constant for the type I acceptor (greater than 50 mM), and displays a high relative specific activity towards the type II substrate (Table 3). The chimeric enzyme Fuc-TC19 is therefore similar to wild type Fuc-TVI, and to the other "Fuc-TVI-like" chimera Fuc-TC4 analyzed *in vitro*.

The complementary chimera Fuc-TC20 (subdomains 4 and 5 from Fuc-TIII transposed into the corresponding position in Fuc-TVI) utilizes neutral and sialylated type I molecules, as well as neutral type II molecules, *in vivo*, to generate Le<sup>a</sup> (6.3% positive cells), sLe<sup>a</sup> (3.3% positive cells) and Le<sup>x</sup> (5.4% positive cells) antigens. Western blot analyses indicate that Fuc-TC20 accumulates in COS-7 cells to levels nearly equivalent to those observed when wild type Fuc-TVI is expressed in this cell line. *In vitro* fucosyltransferase assays demonstrate that this chimeric enzyme maintains an inherently low specific activity towards the type I and type II acceptor substrates examined (apparent  $K_m$ 's exceeding 50 mM; Table 3). However, Fuc-TC20 utilizes the type I acceptor substrate much more efficiently than it does the type II acceptor (Table 3), and thus maintains the *in vitro* type I

acceptor substrate preference exhibited by wild type Fuc-TIII. These results are analogous to those obtained with Fuc-TC18, above. They indicate that the low level of surface antigen expression determined by Fuc-TC20 is due at least in part to an inherently diminished general specific activity. However, they also indicate that this enzyme, like Fuc-TC18 above, has acquired a Fuc-TIII-like phenotype by virtue of subdomains 4 and 5 derived from Fuc-TIII.

Fuc-TC20 is unusual in that it does not yield cell surface sialyl Lewis x expression, but does direct expression of the other three fucosylated cell surface antigens (Fig. 12b). Given that this chimera differs from Fuc-TC18 only by the absence of a potential asparagine-linked glycosylation site within subdomain 3, the observations derived from these two chimeras suggested the possibility that display of an asparagine-linked oligosaccharide by subdomain 3, at least in the context of an otherwise intact Fuc-TVI scaffold, enables catalysis of sialyl Lewis x formation, whereas its absence disables sialyl Lewis x formation. This possibility is supported by the results obtained with chimeras Fuc-TC1 and Fuc-TC2 (Fig. 10), which determine a glycosylation phenotype analogous to that of Fuc-TC20 (Fig. 12b). In chimeras Fuc-TC1 and Fuc-TC2, absence of an asparagine-linked oligosaccharide modification site in subdomain 3, in the context of a full (Fuc-TC2) or partial (Fuc-TC1) COOH-terminal Fuc-TVI scaffold, also correlates with an inability to determine cell surface sialyl Lewis x formation. However, we note that Fuc-TC12

(Fig. 11b), representing a fully wild type Fuc-TVI sequence, except for absence of the asparagine-linked glycosylation site in subdomain 3, determines robust expression of the cell surface sialyl Lewis x antigen (Fig. 11b). Clearly then, the unusual phenotype exhibited by chimeras Fuc-TC20, Fuc-TC1, and Fuc-TC2, cannot be explained by the simple absence of a single asparagine-linked glycosylation site.

In aggregate, the results obtained with the recombinant fucosyltransferase chimeras derived from Fuc-TIII and Fuc-TVI indicate that some complement of the 21 non-identical amino acid residues encompassed within subdomains 4 and 5 of Fuc-TIII and Fuc-TVI (Fig. 9b) can determine whether or not these enzymes efficiently utilize type I oligosaccharide acceptor substrates in COS-7 cells, and strongly influence their ability to discriminate between type I and type II acceptors *in vitro*.

Subdomains 4 and 5 from Fuc-TV leave intact the type I substrate utilization activity of Fuc-TIII - A third human  $\alpha(1,3)$ Fuc-T, termed Fuc-TV (Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160), shares enzymatic properties with both Fuc-TIII and Fuc-TVI, and is very similar in its primary sequence to these enzymes. Specifically, Fuc-TV determines a cell surface glycosylation phenotype similar to that generated by Fuc-TVI when examined in transfected cell lines (Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160), but can also utilize type I acceptor substrates at detectable efficiencies, when tested *in vitro* with low

molecular weight acceptor substrates (Weston, B.W., et al (1992) *J. Biol. Chem.*, vol. 267, 4152-4160; Weston, B. W., et al (1992) *J. Biol. Chem.*, vol. 267, 24575-24584). Its primary sequence across subdomains 4 and 5 is equally similar to both Fuc-TIII and Fuc-TVI (34 identical residues across spans of 47; Fig. 13). This enzyme and its properties therefore present an opportunity to further define type I substrate utilization-determining amino acid residues within subdomains 4 and 5, by creating and testing a chimera in which subdomains 4 and 5 from Fuc-TV are exchanged with the corresponding domains in Fuc-TIII. The results of these manipulations indicate that the robust type I substrate utilization is maintained when subdomains 4 and 5 within Fuc-TIII are substituted with the corresponding subdomains from Fuc-TV (Fig. 12a). These results, when considered together with those obtained with the chimeras derived from Fuc-TIII and Fuc-TVI, indicate that the type I acceptor substrate utilization properties of these enzymes can be controlled by a quite limited subset of the 21 non-identical amino acid residues suggested to be important by the Fuc-TIII:Fuc-TVI chimera experiments (Fig. 13).



These experiments demonstrate that utilization of type I oligosaccharide substrates by the human  $\alpha(1,3)$ fucosyltransferases Fuc-TIIII, Fuc-TV, and Fuc-TVI is determined in large measure by a relatively short peptide segment, encompassed by subdomains 4 and 5, and localized to the  $\text{NH}_2$ -terminus of each enzyme's Golgi lumenal domain. Examination of the residues within this segment, in each enzyme, suggests the simple possibility that, in the context of a "generic"  $\alpha(1,3)$ fucosyltransferase background, efficient type I substrate utilization is allowed by residues shared by Fuc-TIIII and Fuc-TV and found within subdomains 4 and 5, whereas type I substrate utilization is "disabled" by residues in this segment that are unique to Fuc-TVI.

In the context of this simple model, the more obvious "Fuc-TVI-unique" amino acid residues (Fig. 13) that might operate singly, or in concert with other neighboring residues, to disable type I acceptor substrate utilization, include the non-conservative differences represented by: (i) the arginine residue at position 110 in Fuc-TVI, that corresponds to a tryptophan residue that occupies the analogous position in both Fuc-TIIII and Fuc-TV; (ii) a pair of arginine residues in Fuc-TVI at positions (122 and 126) that correspond to a pair of proline residues in Fuc-TIIII and Fuc-TV; (iii) a tryptophan residue (143) in Fuc-TVI that corresponds to glutamine or arginine in Fuc-TIIII or Fuc-TV, respectively; and (iv) a lysine residue at position 146 in Fuc-TVI, that corresponds to a glutamic acid residue in Fuc-TIIII and Fuc-TV. It is also

possible that type I acceptor substrate utilization may be influenced by the more conservative amino acid sequence differences identified in Figure 13.

Although these studies have identified candidate residues for acceptor substrate discrimination, they do not allow us to discern the mechanism(s) through which these residues operate in this process. However, previous work by others in this area suggests a role for hydrogen bonding interactions in acceptor substrate binding and utilization. For example, in an evaluation of deoxygenated oligosaccharide acceptor substrates for two different human fucosyltransferases (corresponding to Fuc-TIIII and Fuc-TVI), Hindsgaul and colleagues report that acceptor substrates deoxygenated at the reactive hydroxyl position do not act as inhibitors of these enzymes (Hindsgaul, O., et al (1991) *J. Bio. Chem.*, vol. 266, 17858-17862). Hindsgaul et. al. interpret these results to mean that these fucosyltransferases, as well as other glycosyltransferases, bind to their acceptor substrates, and subsequently effect catalysis, through hydrogen bonding interactions between the reactive hydroxyl group on the acceptor and one or more basic groups in the enzyme (Hindsgaul, O., et al (1991) *J. Bio. Chem.*, vol. 266, 17858-17862). In the context of this hypothesis, we note that, in aggregate, the "Fuc-TVI-specific" residues shown in Figure 13 are largely basic in character, relative to the corresponding residues in Fuc-TIIII and Fuc-TV. The non-conserved tryptophan residues in subdomains 4 and 5, and other such polar residues,

may also mediate fucosyltransferase acceptor substrate recognition, again presumably through hydrogen bonding interactions, as suggested from studies of  $\beta(1,4)$ galactosyltransferase (Aoki, D., et al (1990) *EMBO J.*, vol. 9, 3171-3178). Whether or not these residues operate to determine binding to, and fucosylation of, type II acceptor substrates exclusively (and perhaps at the expense of type I acceptor substrate binding) remains to be experimentally determined.

On a more global scale, it seems probable that fucosyltransferase-specific substrate recognition and modification properties will also be found to be more than simply a function of the ability of the relevant subdomains to "bind" to the oligosaccharide acceptor. For instance, we can anticipate that some of the residues in these subdomains may interact in space with amino acids far away along the linear sequence of these enzyme, to alter acceptor substrate utilization. Alternatively, residues in these subdomains may influence acceptor substrate recognition by residues elsewhere in the molecule, via transmitted conformational change dictated by the specific residues within these subdomains. Finally, it is possible that subdomain-dependent proteolytic events indirectly determine acceptor substrate specificity by controlling the liberation of distinct soluble fucosyltransferase fragments with different acceptor substrate specificities.

It is emphasized that the present studies have focused on detecting the products of these enzymes at a relatively low level of resolution (i.e., at the level of cell surface antigen expression). This approach is advantageous in that it measures product(s) formed by an enzyme within its correct cellular environment. Furthermore, since the fraction of antigen-positive cells we observe correlates very well with catalytic activities measured *in vitro*, this parameter seems to accurately reflect the biochemical nature of the enzyme under study. By contrast, the mean fluorescent intensities of the antigen-positive cells fall within a similar range, regardless of the enzyme analyzed, and do not correlate with *in vitro* activity. We do not understand this phenomenon, although we speculate that this can be accounted for by the possibility that acceptor substrate concentration and/or accessibility within COS cells is limiting to the point that utilization of the small amounts of the available acceptor substrate molecules yields a virtually maximal, though easily detectable, signal. The broad spectrum of enzyme protein expression level obtained within a population of transiently transfected cells may play upon such limiting substrate levels to yield an off-on, or digital signal response that is a function of the efficiency of the enzyme under study. For example, in a population of cells expressing an enzyme with robust inherent activity, many cells, including those that express a relatively small amount of enzyme protein, will contain sufficient intracellular enzyme activity to convert

them to antigen-positivity (hence the relatively larger fraction of antigen-positive cells seen with efficient enzymes), even though the limiting amounts of substrate operate to place a strict upper boundary on the "brightness" of such cells. By contrast, in a population of cells expressing an enzyme with a low inherent activity, only the relatively small fraction of cells that have accumulated relatively large amounts of enzyme protein will contain sufficient enzyme activity to exceed the threshold for substrate conversion (hence the relatively small fraction of antigen positive cells with inefficient enzymes), and will become antigen-positive, with a mean fluorescence intensity that can be roughly maximal. Additional work will be required to confirm or refute this notion.

The present approach cannot tell much about the nature of the glycoconjugate(s) that displays the fucosylated antigens, or about the structures of the glycoconjugate precursors that serve as substrates for these enzymes within these cells (aside from the fact that some of these moieties terminate with neutral and  $\alpha 2 \rightarrow 3$ sialylated type I and type II structures). It will be important to eventually know this information, however, since it is possible that some of the apparent subdomain-dependent acceptor substrate specificity we observe is also a function of the type of glycoconjugate that displays the acceptor di- or tri-saccharide moieties with which we have concerned ourselves here. It is noted that there are well known precedents for this type of

cis-modulation of glycosyltransferase substrate specificity by the underlying glycoprotein sequence (Kornfeld, S. (1987) *FASEB J.*, vol. 1, 462-468; Baenziger, J.U. (1994) *FASEB J.*, vol. 8, 1019-1025).

Table 3. In vitro apparent  $K_m$ 's and relative fucosyltransferase activities of recombinant Fuc-T chimeras.

<u>Fucosyltrans- ferase</u>	<u>Apparent <math>K_m</math> LacNAc, mM</u>	<u>Apparent <math>K_m</math> LNB-I, mM</u>	<u>Specific Activity Ratio LacNAc/LNB-I</u>
Fuc-TIII	8.1	12.7	0.12
Fuc-TVI	4.9	>50	2610
Fuc-TC2	>50	>50	0.03
Fuc-TC4	5.8	>50	3646
Fuc-TC18	>50	>50	0.05
Fuc-TC19	1.1	>50	189
Fuc-TC20	>50	>50	0.04

The structures of the present chimeric enzymes are shown in Table 4.

TABLE 4

<u>Chimera Name</u>	<u>Fuc-T and component amino acid residues used</u>
Fuc-TC1	Fuc-TIII(1-301) plus Fuc-TVI(301-359)
Fuc-TC2	Fuc-TIII(1-160) plus Fuc-TVI(160-359)
Fuc-TC3	Fuc-TVI(1-300) plus Fuc-TIII(302-361)
Fuc-TC4	Fuc-TVI(1-159) plus Fuc-TIII(161-361)
Fuc-TC5	Fuc-TIII(1-33) plus Fuc-TVI(34-69) plus Fuc-TIII (71-361)
Fuc-TC6	Fuc-TIII(1-70) plus Fuc-TVI(70-85) plus Fuc-TIII (87-361)
Fuc-TC7	Fuc-TIII(1-86) plus Fuc-TVI(86-98) plus Fuc-TIII (100-361)
Fuc-TC8	Fuc-TIII(1-99) plus Fuc-TVI(99-129) plus Fuc-TIII (131-361)
Fuc-TC9	Fuc-TIII(1-130) plus Fuc-TVI(130-159) plus Fuc-TIII (161-361)
Fuc-TC10	Fuc-TVI(1-33) plus Fuc-TIII(34-70) plus Fuc-TVI (70-359)
Fuc-TC11	Fuc-TVI(1-69) plus Fuc-TIII(71-86) plus Fuc-TVI (86-359)
Fuc-TC12	Fuc-TVI(1-85) plus Fuc-TIII(87-99) plus Fuc-TVI (99-359)
Fuc-TC13	Fuc-TVI(1-98) plus Fuc-TIII(100-130) plus Fuc-TVI (130-359)
Fuc-TC14	Fuc-TVI(1-129) plus Fuc-TIII(131-160) plus Fuc-TVI (160-359)
Fuc-TC15	Fuc-TIII(1-33) plus Fuc-TVI(34-98) plus Fuc-TIII(100-361)
Fuc-TC16	Fuc-TIII(1-86) plus Fuc-TVI(86-159) plus Fuc-TIII(161-361)
Fuc-TC17	Fuc-TVI(1-33) plus Fuc-TIII(34-99) plus Fuc-TVI(99-359)
Fuc-TC18	Fuc-TVI(1-85) plus Fuc-TIII(87-160) plus Fuc-TVI(160-359)
Fuc-TC19	Fuc-TIII(1-99) plus Fuc-TVI(99-159) plus Fuc-TIII(161-361)
Fuc-TC20	Fuc-TVI(1-98) plus Fuc-TIII(100-160) plus Fuc-TVI(160-359)
Fuc-TC21	Fuc-TIII(1-99) plus Fuc-TV(116-166) plus Fuc-TIII(161-361)

Lyophilized plasmids containing DNA encoding these polypeptides were deposited with the ATCC in Rockville, Maryland on September 7, 1995.

U.S. Application Serial Number 08/393,246, filed on February 23, 1995, is incorporated herein by reference, in its entirety.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: LOWE, JOHN B.
- (ii) TITLE OF INVENTION: METHODS AND PRODUCTS FOR THE SYNTHESIS OF OLIGOSACCHARIDE STRUCTURES ON GLYCOPROTEINS, GLYCOLIPIDS, OR AS FREE MOLECULES, AND FOR THE ISOLATION OF CLONED GENETIC SEQUENCES THAT DETERMINE THESE STRUCTU
- (iii) NUMBER OF SEQUENCES: 14
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C.
  - (B) STREET: 1755 Jefferson Davis Highway, Fourth Floor
  - (C) CITY: Arlington
  - (D) STATE: Virginia
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 22202
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE: 20-JUL-1992
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Lavalleye, Jean-Paul M. P.
  - (B) REGISTRATION NUMBER: 31,451
  - (C) REFERENCE/DOCKET NUMBER: 2363-060-55
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (703)521-4500
  - (B) TELEFAX: (703)486-2347
  - (C) TELEX: 248855 OPAT UR

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2043 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown

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(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGGAAACCTG CCATGGCCTC CTGGTGAGCT GTCCTCATCC ACTGCTCGCT GCCTCTCCAG	60
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CTGGCCGCAC TGCTATTTCA GCTGCTGGTG GCTGTGTGTT TCTTCTCCTA CCTGCGTGTG	180
TCCCGAGACG ATGCCACTGG ATCCCCTAGG GCTCCCAGTG GGTCTCCCG ACAGGACACC	240
ACTCCCACCC GCCCCACCCT CCTGATCCTG CTATGGACAT GGCCTTTCCA CATCCCTGTG	300
GCTCTGTCCC GCTGTTTACA GATGGTGCCC GGCACAGCCG ACTGCCACAT CACTGCCGAC	360
CGCAAGGTGT ACCCACAGGC AGACACGGTC ATCGTGCACC ACTGGGATAT CATGTCCAAC	420
CCTAAGTCAC GCCTCCCACC TTCCCCGAGG CCGCAGGGGC AGCGCTGGAT CTGGTTCAAC	480
TTGGAGCCAC CCCCTAACTG CCAGCACCTG GAAGCCCTGG ACAGATACTT CAATCTCACC	540
ATGTCCTACC GCAGCGACTC CGACATCTTC ACGCCCTACG GCTGGCTGGA GCCGTGGTCC	600
GGCCAGCCTG CCCACCCACC GCTCAACCTC TCGGCCAAGA CCGAGCTGGT GGCCTGGGCG	660
GTGTCCAAC TGAAGCCGGA CTCAGCCAGG GTGCGCTACT ACCAGAGCCT GCAGGCTCAT	720
CTCAAGGTGG ACGTGTACGG ACGCTCCCAC AAGCCCCTGC CCAAGGGGAC CATGATGGAG	780
ACGCTGTCCC GGTACAAGTT CTACCTGGCC TTCGAGAACT CCTTGCACCC CGACTACATC	840
ACCGAGAAGC TGTGGAGGAA CGCCCTGGAG GCCTGGGCCC TGCCCGTGGT GCTGGGCCCC	900
AGCAGAAGCA ACTACGAGAG GTTCCTGCCA CCCGACGCCT TCATCCACGT GGACGACTTC	960
CAGAGCCCCA AGGACCTGGC CCGGTACCTG CAGGAGCTGG ACAAGGACCA CGCCCGCTAC	1020
CTGAGCTACT TTCGCTGGCG GGAGACGCTG CGGCCTCGCT CCTTCAGCTG GGCACTGGAT	1080
TTCTGCAAGG CCTGCTGGAA ACTGCAGCAG GAATCCAGGT ACCAGACGGT GCGCAGCATA	1140
GCGGCTTGGT TCACCTGAGA GGCCGGCATG GTGCCTGGGC TGCCGGGAAC CTCATCTGCC	1200
TGGGGCCTCA CCTGCTGGAG TCCTTTGTGG CCAACCCTCT CTCTTACCTG GGACCTCACA	1260

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CGCTGGGCTT CACGGCTGCC AGGAGCCTCT CCCCTCCAGA AGACTTGCCT GCTAGGGACC 1320  
 TCGCCTGCTG GGGACCTCGC CTGTTGGGGA CCTCACCTGC TGGGGACCTC ACCTGCTGGG 1380  
 GACCTTGGCT GCTGGAGGCT GCACCTACTG AGGATGTCGG CGGTCGGGGA CTTTACCTGC 1440  
 TGGGACCTGC TCCCAGAGAC CTTGCCACAC TGAATCTCAC CTGCTGGGGA CCTCACCTG 1500  
 GAGGGCCCTG GGCCCTGGGG AACTGGCTTA CTTGGGGCCC CACCCGGGAG TGATGGTTCT 1560  
 GGCTGATTG TTTGTGATGT TGTTAGCCGC CTGTGAGGGG TGCAGAGAGA TCATCACGGC 1620  
 ACGGTTTCCA GATGTAATAC TGCAAGGAAA AATGATGACG TGTCTCCTCA CTCTAGAGGG 1680  
 GTTGGTCCCA TGGGTTAAGA GCTCACCCCA GGTTCACACC TCAGGGGTTA AGAGCTCAGA 1740  
 GTTCAGACAG GTCCAAGTTC AAGCCCAGGA CCACCACTTA TAGGGTAÇAG GTGGGATCGA 1800  
 CTGTAAATGA GGACTTCTGG AACATTCCAA ATATTCTGGG GTTGAGGGAA ATTGCTGCTG 1860  
 TCTACAAAAT GCCAAGGGTG GACAGGCGCT GTGGCTCAGC CCTGTAATTC CAGCACTTTG 1920  
 GGAGGCTGAG GTAGGAGGAT TGATTGAGGC CAAGAGTTAA AGACCAGCCT GGTCAATATA 1980  
 GCAAGACCAC GTCTCTAAAT AAAAAATAAT AGGCCGGCCA GGAAAAAAAA AAAAAAAAAA 2040  
 AAA 2043

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 361 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 Leu Ala Ala Leu Leu Phe Gln Leu Leu Val Ala Val Cys Phe Phe Ser  
 20 25 30  
 Tyr Leu Arg Val Ser Arg Asp Asp Ala Thr Gly Ser Pro Arg Ala Pro  
 35 40 45  
 Ser Gly Ser Ser Arg Gln Asp Thr Thr Pro Thr Arg Pro Thr Leu Leu

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50					55					60					
Ile	Leu	Leu	Trp	Thr	Trp	Pro	Phe	His	Ile	Pro	Val	Ala	Leu	Ser	Arg
65					70					75					80
Cys	Ser	Glu	Met	Val	Pro	Gly	Thr	Ala	Asp	Cys	His	Ile	Thr	Ala	Asp
				85					90					95	
Arg	Lys	Val	Tyr	Pro	Gln	Ala	Asp	Thr	Val	Ile	Val	His	His	Trp	Asp
			100					105					110		
Ile	Met	Ser	Asn	Pro	Lys	Ser	Arg	Leu	Pro	Pro	Ser	Pro	Arg	Pro	Gln
			115				120					125			
Gly	Gln	Arg	Trp	Ile	Trp	Phe	Asn	Leu	Glu	Pro	Pro	Pro	Asn	Cys	Gln
	130					135						140			
His	Leu	Glu	Ala	Leu	Asp	Arg	Tyr	Phe	Asn	Leu	Thr	Met	Ser	Tyr	Arg
145					150					155					160
Ser	Asp	Ser	Asp	Ile	Phe	Thr	Pro	Tyr	Gly	Trp	Leu	Glu	Pro	Trp	Ser
				165					170					175	
Gly	Gln	Pro	Ala	His	Pro	Pro	Leu	Asn	Leu	Ser	Ala	Lys	Thr	Glu	Leu
			180					185					190		
Val	Ala	Trp	Ala	Val	Ser	Asn	Trp	Lys	Pro	Asp	Ser	Ala	Arg	Val	Arg
			195				200					205			
Tyr	Tyr	Gln	Ser	Leu	Gln	Ala	His	Leu	Lys	Val	Asp	Val	Tyr	Gly	Arg
	210					215					220				
Ser	His	Lys	Pro	Leu	Pro	Lys	Gly	Thr	Met	Met	Glu	Thr	Leu	Ser	Arg
225					230					235					240
Tyr	Lys	Phe	Tyr	Leu	Ala	Phe	Glu	Asn	Ser	Leu	His	Pro	Asp	Tyr	Ile
				245					250					255	
Thr	Glu	Lys	Leu	Trp	Arg	Asn	Ala	Leu	Glu	Ala	Trp	Ala	Val	Pro	Val
			260					265					270		
Val	Leu	Gly	Pro	Ser	Arg	Ser	Asn	Tyr	Glu	Arg	Phe	Leu	Pro	Pro	Asp
		275					280					285			
Ala	Phe	Ile	His	Val	Asp	Asp	Phe	Gln	Ser	Pro	Lys	Asp	Leu	Ala	Arg
						295					300				
Tyr	Leu	Gln	Glu	Leu	Asp	Lys	Asp	His	Ala	Arg	Tyr	Leu	Ser	Tyr	Phe
305					310					315					320
Arg	Trp	Arg	Glu	Thr	Leu	Arg	Pro	Arg	Ser	Phe	Ser	Trp	Ala	Leu	Asp

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	325		330		335										
Phe	Cys	Lys	Ala	Cys	Trp	Lys	Leu	Gln	Gln	Glu	Ser	Arg	Tyr	Gln	Thr
	340						345						350		
Val	Arg	Ser	Ile	Ala	Ala	Trp	Phe	Thr							
	355						360								

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1500 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTTCCCTTG TAGACTCTTC TTGGAATGAG AAGTACCGAT TCTGCTGAAG ACCTCGCGCT	60
CTCAGGCTCT GGGAGTTGGA ACCCTGTACC TTCCTTTCCT CTGCTGAGCC CTGCCTCCTT	120
AGGCAGGCCA GAGCTCGACA GAACTCGGTT GCTTTGCTGT TTGCTTTGGA GGGAACACAG	180
CTGACGATGA GGCTGACTTT GAACTCAAGA GATCTGCTTA CCCCAGTCTC CTGGAATTAA	240
AGGCCTGTAC TACATTTGCC TGGACCTAAG ATTTTCATGA TCACTATGCT TCAAGATCTC	300
CATGTCAACA AGATCTCCAT GTCAAGATCC AAGTCAGAAA CAAGTCTTCC ATCCTCAAGA	360
TCTGGATCAC AGGAGAAAAT AATGAATGTC AAGGGAAAAG TAATCCTGTT GATGCTGATT	420
GTCTCAACCG TGGTTGTCGT GTTTTGGGAA TATGTCAACA GAATTCCAGA GGTGCGTGAG	480
AACAGATGGC AGAAGGACTG GTGGTTCCCA AGCTGGTTTA AAAATGGGAC CCACAGTTAT	540
CAAGAAGACA ACGTAGAAGG ACGGAGAGAA AAGGGTAGAA ATGGAGATCG CATTGAAGAG	600
CCTCAGCTAT GGGACTGGTT CAATCCAAAG AACCGCCCGG ATGTTTTGAC AGTGACCCCG	660
TGGAAGGCGC CGATTGTGTG GGAAGGCACT TATGACACAG CTCTGCTGGA AAAGTACTAC	720
GCCACACAGA AACTCACTGT GGGGCTGACA GTGTTTGCTG TGGGAAAGTA CATTGAGCAT	780
TACTTAGAAG ACTTTCTGGA GTCTGCTGAC ATGTACTTCA TGGTTGGCCA TCGGGTCATA	840

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TTTTACGTCA TGATAGACGA CACCTCCCGG ATGCCTGTCTG TGCACCTGAA CCCTCTACAT      900
TCCTTACAAG TCTTTGAGAT CAGGTCTGAG AAGAGGTGGC AGGATATCAG CATGATGCGC      960
ATGAAGACCA TTGGGGAGCA CATCCTGGCC CACATCCAGC ACGAGGTCGA CTTCTCTTTC      1020
TGCATGGACG TGGATCAAGT CTTTCAAGAC AACTTCGGGG TGGAAACTCT GGGCCAGCTG      1080
GTAGCACAGC TCCAGGCCTG GTGGTACAAG GCCAGTCCCG AGAAGTTCAC CTATGAGAGG      1140
CGGGAAGTGT CGGCCCGGTA CATTCCATTC GGAGAGGGGG ATTTTACTA CCACGCGGCC      1200
ATTTTGGAG GAACGCCTAC TCACATTCTC AACCTCACCA GGGAGTGCTT TAAGGGGATC      1260
CTCCAGGACA AGAAACATGA CATAGAAGCC CAGTGGCATG ATGAGAGCCA CCTCAACAAA      1320
TACTTCCTTT TCAACAAACC CACTAAAATC CTATCTCCAG AGTATTGCTG GGACTATCAG      1380
ATAGGCCTGC CTTGAGATAT TAAAAGTGTC AAGGTAGCTT GGCAGACAAA AGAGTATAAT      1440
TTGGTTAGAA ATAATGTCTG ACTTCAAATT GTGATGGAAA CTTGACACTA TTTCTAACCA      1500

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## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 394 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Ile Thr Met Leu Gln Asp Leu His Val Asn Lys Ile Ser Met Ser
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Arg Ser Lys Ser Glu Thr Ser Leu Pro Ser Ser Arg Ser Gly Ser Gln
20           25           30
Glu Lys Ile Met Asn Val Lys Gly Lys Val Ile Leu Leu Met Leu Ile
35           40           45
Val Ser Thr Val Val Val Val Phe Trp Glu Tyr Val Asn Arg Ile Pro
50           55           60
Glu Val Gly Glu Asn Arg Trp Gln Lys Asp Trp Trp Phe Pro Ser Trp
65           70           75           80

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Phe Lys Asn Gly Thr His Ser Tyr Gln Glu Asp Asn Val Glu Gly Arg  
 85 90 95  
 Arg Glu Lys Gly Arg Asn Gly Asp Arg Ile Glu Glu Pro Gln Leu Trp  
 100 105 110  
 Asp Trp Phe Asn Pro Lys Asn Arg Pro Asp Val Leu Thr Val Thr Pro  
 115 120 125  
 Trp Lys Ala Pro Ile Val Trp Glu Gly Thr Tyr Asp Thr Ala Leu Leu  
 130 135 140  
 Glu Lys Tyr Tyr Ala Thr Gln Lys Leu Thr Val Gly Leu Thr Val Phe  
 145 150 155 160  
 Ala Val Gly Lys Tyr Ile Glu His Tyr Leu Glu Asp Phe Leu Glu Ser  
 165 170 175  
 Ala Asp Met Tyr Phe Met Val Gly His Arg Val Ile Phe Tyr Val Met  
 180 185 190  
 Ile Asp Asp Thr Ser Arg Met Pro Val Val His Leu Asn Pro Leu His  
 195 200 205  
 Ser Leu Gln Val Phe Glu Ile Arg Ser Glu Lys Arg Trp Gln Asp Ile  
 210 215 220  
 Ser Met Met Arg Met Lys Thr Ile Gly Glu His Ile Leu Ala His Ile  
 225 230 235 240  
 Gln His Glu Val Asp Phe Leu Phe Cys Met Asp Val Asp Gln Val Phe  
 245 250 255  
 Gln Asp Asn Phe Gly Val Glu Thr Leu Gly Gln Leu Val Ala Gln Leu  
 260 265 270  
 Gln Ala Trp Trp Tyr Lys Ala Ser Pro Glu Lys Phe Thr Tyr Glu Arg  
 275 280 285  
 Arg Glu Leu Ser Ala Ala Tyr Ile Pro Phe Gly Glu Gly Asp Phe Tyr  
 290 295 300  
 Tyr His Ala Ala Ile Phe Gly Gly Thr Pro Thr His Ile Leu Asn Leu  
 305 310 315 320  
 Thr Arg Glu Cys Phe Lys Gly Ile Leu Gln Asp Lys Lys His Asp Ile  
 325 330 335  
 Glu Ala Gln Trp His Asp Glu Ser His Leu Asn Lys Tyr Phe Leu Phe  
 340 345 350

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Asn Lys Pro Thr Lys Ile Leu Ser Pro Glu Tyr Cys Trp Asp Tyr Gln  
                   355                                  360                                  365

Ile Gly Leu Pro Ser Asp Ile Lys Ser Val Lys Val Ala Trp Gln Thr  
           370                                  375                                  380

Lys Glu Tyr Asn Leu Val Arg Asn Asn Val  
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## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8174 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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GGTGAAAGGG GCTGGGGGCC CACATTCCTG AGTCTCAGAG AGAAGGATCT GGGGTCTCAA	180
GCACCTGAGT CGGAGGGAGG AGGGGTGCTG GGCTCCTGGA AAAACCACCT CTTGGACCAT	240
CTATGCAGAT CACGCAGAAC AAGAGAAATT TCTGCGCCCC ATCTGAATTT CTAAGTTTGG	300
GGGGAGGGCG TGATCTGACA CTGAGGTTCC TTGATCCTCA GCAAGGCGGC AATTGCTGTA	360
TGAAAGAAGC GACCGCATCT GAGACACAAG TATCCTGCCT TGGAAGCCTC TCACCTGGCC	420
GTGGGGCAAC CTCAACCTCA TCTGTCCCTG CTCAGATGCT CAGACCCTGG ACATCCCAGC	480
CTCCTCCTCC CTGATGCAAT CCTGGTGTTT CTTTCACCAG AGAAGCCATC CCAGGCCCAG	540
GCAGGTGCTC CTGAAATAAC CTGGGGGGAG GGGTGGCTGA AAGTCCCTGA CTGGAGTTGG	600
CAGCCAAGCC AGGCCCTGGA GTGGGCACCC AGAGGGAAGA CAGGTGGCT AATTTCCTGG	660
AGCCCCTAAG GGTGCAAGGG TAGGCCTTCT GTGTCTGAGG GAGGAGGGCT GGGGCTCTGG	720
ACTCCTGGGT CTGAGGGAGG AGGGGTGGGG GGCCTGGACT CCTGGGTCTG AGGGAGGAGG	780



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GTCTGGGCCT GTACTCCTGG ATCTGAGGGA GGAGGGGCTG GGGAACTTGG GCTCCTGGGT 840  
CTGAGGGAGG AGGGAGCTTT GGTCTGGACT CCTGGGTCTG AGGGAGTAGG GGCTAGGGAT 900  
CTGGACTCGT GGGTGTGAGG AAGGAGGGGC TGGGGTCCTG GACTCCTGGG TCTGAGGAAG 960  
GAGGGGCAGG GGGCTTGGAC TCCTGGGTCT GAGGAAGGAG GGGCCGGGAG CCTGGACTCC 1020  
TAAGTCTGAG GGAGGAGGGT CTGGGGGCCT GGA CTGCTGG GTGTGAGCAG AAGGGTCTGG 1080  
GTGCTGGGAG TCCCGAGCCT GGGGAGATGA TGGTTAACT TCTGGGAATC AAGTCAAAC 1140  
CCTGAGTCTT TGACATTGAT GTATCTTGAA TGGGAGGGTC AGTCTGTGGG GAAGGATTAC 1200  
CCAGGTGCCG AGGCAAGAGA CTGAAGGCAC AAAC TGTTC AGTATAATAA AGAAAATAGT 1260  
TAGAATAAGA ATAGTTATCA TACAAATTAG ATATAGAGAT GATCATGGAC AGTATCAATC 1320  
ATTAGTGTA ACATTATTAA TCATTAGCTA TTA CTTTAT TCTTTGTGT ATA ACTAATA 1380  
TAACCAGGAA ACAACCGGTG GGTATAGGGT CAGGTACTGA AGGGACATTG TGAGAAGTGA 1440  
CCTAGAAGGC AAGAGGTGAG CCTTCTGTCA CACCGGCATA AGGGCCTCTT GAGGGCTCCT 1500  
TGGTCAAGCG GGAACGCCAG TGTCTGGGAA GGCACCCGTT ACTCAGCAGA CCACGAAAGG 1560  
GAATCTCCTT TTCTTGAGG AGTCAGGGAA CACTCTGCTC CACCAGCTTC TTGTGGGAGG 1620  
CTGGGTATTA TCTAGGCCTG CCCGCAGTCA TCCTGCTGTG CTGTGCTTCA ATGGTCACGC 1680  
TCCTTGTCTT CTGCAATTTT CCTCCCGTAC TCCTGGTTCC TCTTTGAAAG TCGTAGTAGA 1740  
TAGCGGTAGA AGAAATAGTG AAAGCCTTTT TTTTTTTTTT TTTGAGGCGG AGTCTCGCTC 1800  
TGTCCCCCAG GCTGGAGTGC AGTGGCGTGA TCTCGGCTCA CTGCAATCTC CGCCTCCTGG 1860  
GTTACACCA TTCTCCTGCC TCACCCTCCC AAATAGCTAG GACTACAGGC GCCCTCCACC 1920  
ACGCGCCCGG ATAATTTTTT GTATTTTTTAG TAGAGACAGG GTTTCACCGT GTTAGCCAGG 1980  
ATGGCCTCCA CTCCTGACC TTGTGATCCG CCCGCCTCAG CCTCCCAAAG TGCTGGGATT 2040  
ACAGGCGTGA GCCACCGCGC CCGCCCGAAA TAGTGAAAGT CTAAAGTCT TTGATCTTTC 2100  
TTATAAGTGC AGAGAAGAAA ACGCTGACAT ATGCTGCCTT CTCTTTCTGC TTCGGCTGCC 2160  
TAAAAGGGAA GGGCCCCCTG TCCCATGATC ACGTGA CTG CTTGACCTTA TCAGTCATTT 2220  
GGACGACTCA CCCTCCTTAT CCTGCCCCC CTTGTCTTGT ATACAATAAA TATCAGCGCG 2280  
CCCAGCCATT CGGGGCCACT ACCGGTCTCT GCGTCTTGAT GGTAGTGGTC CCCC GGCCCC 2340

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AGCTGTTTTTCTCTTATCTCTTTGTCTTGTGTCTTTATTTCTTACAATCTCTCCTCTCCT	2400
CACAGGGGAA GAACACCCAC CCGCAAAGCC CCGTAGGGCT GGACCCTACG TTAGCCTGCC	2460
CTGCTCGGGG TTGGCGATGC TGGAGGTGGG CCTTGGACCA GAGAAAATGC TTTAATTAGG	2520
TGACAAGCGG GCAGAGGCCT TTGTCTCTGG CGCCGGCAGC CACGGCCCCC GCTGACGGCG	2580
TGGGAAACAG ACCCTGTTCC ACTCCGGTCT CCAGCCTTGG AATGGTTGCC TTCGTGCAGT	2640
GCAGGTCTGG AAAGTAGCAG TTTGGCACGG GACCCTAGAA TTCCCCAAAA GGAGTGAATA	2700
GGGGCTGGGA TTCTGGAATT TGAGTGTGGA CGGTGAGGCG GGGGGTGTGG GAGATCGGAG	2760
ACCCTGGTGG GCGCGGGAGC ACCTGCAGGC TGGAGGCCCT CGCGCGCTCC GGCGGCAGCC	2820
TGGCAAACAG GTTCTCCATC CCCCAGGAGG ACGCGGCAGA GGGCGGACGA TCGCTCCACT	2880
CGCCGGGACC AGGTGCGGGG GCCCTGCCCA GCCGCTGGGG CGTGGCCAGG CTCGAAGCAC	2940
CCAGGTGTGC GGGGCCGACT CTAAGCCCTG GCACCGGAAG AGAGAGGGCG GCGGATTGGA	3000
CCTCCCGGCT CCAGCATTGC AACTGGGCGC TCCGTCTCCT GGTCCACGCA ATGATGCTGC	3060
GGCTGCTCAG AAGCCAGGTA GCCTGCCCTG GGTGAAGCCT TCGCGCAGGT CAATGACGGG	3120
GCGGAGGGGC AGGGCGCGGT CCCCTGCATC CCCGATCTGG GGAGCGGTGG GCCCAGGGGC	3180
CATCGCCTTA GCCCCTGGCG CTGGGGCTCG GCGCCAAGTG ACGGGCGGGG CTCCACCTTC	3240
CAGCCATCCG CCCGGCCCCG GAGGGCGGAC GCTGCGAGAC TCCCGGCCGC GCCCTCTCCT	3300
TCCTCTCCTC CCAAAGCCCT CGCTGCCAGT CCGGACAGGC TCGCGGAGG GGAGGGCTGC	3360
CGGGCCGGAT AGCCGGACGC CTGGCGTTCC AGGGGCGGCC GGATGTGGCC TGCCTTTGCG	3420
GAGGGTGC GC TCCGGCCAG AAAAGCGGAC TGTGGATCTG CCACCTGCAA GCAGCTCGGG	3480
TAAGTGGGGA CTGCCCCACT CAGTTGTTCC TGGGACCCAG GAACAACTCC TTCAGAACCA	3540
GGAGGTGCAC CCCCACCTC TTCTCCAGGT CTTCTTAAG CCCTAGGAAT CTCCGCCACC	3600
TCCCCAGCCA TTAATCCTCC AGGAACCAAG ATGCTCCTTC CGCTCCTGAC CCTCCAGCCT	3660
CTCTTGTTTT ACTTGAACTA TCGTTTCCCA TCACCACCTC TGTGGTGGAT TTTGCGCCTC	3720
ACAGACAGGT ACTCCTGAGA AACAGGCTGG TGAAGAGTC CAGTATCAGC GGAACCTACA	3780
GGAGGGGAGA CTCGAGATTC CTTCAGGAAA GGTGTAGGAA CCTGGACCAC TTTCTTTTTT	3840
TTTTTTTTTT TTTTTTTAAG ACAGGGTCCC TCTCTGTGCG GCAAGCTGGA GTGCAGTCAG	3900

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CGGTGCTATC GCGGCTCATT GTGAGCTCCG GGGATCCTCC CGCCTTAGCA TCCGGTGTAG	3960
CTGAGACCAC AGACATGTGC CACCATGCCA AGCTAATTTT ATTTATTTTT TTTTGGAGAC	4020
GGAGTTTCAC TCTTGTTGCC CAGGCTGGAG TGTAAATGGCA TGATCTCAGC TCACCGCAAC	4080
TCCCGCCCCC CGGGTTCAGG CGATTCTCCT GCCTCAGCCT CCCGAGTGGC TGGGATTACA	4140
GGCATGCGCC ACCATGCCCC GCTAATTTTG TATTTTAAGT AGAGACAGGG TTTCTCCACG	4200
TTGGTCAGGC TGGTCTCGAA CTCCCAACCT CAGGTGATCC ACCCACCTTG GCCTCCCAAA	4260
GTGCTGGGAT TACAGGTGTG AGCCACCGCG CCTGGCCCAT GCCAAGCTAA TTTTAAATTT	4320
TTTTTGTAAG AGTGCTCTGT TGCCCAGGCT GATCTTGAAC TCCTGGGCTC AAGGGATCCT	4380
CCCATCTCAG CCTCCCAATA TGCTGGGATT ACAGGTGTGA GCCACAGTGC CCAGCCAAAC	4440
CATGGCTATC TTGAAAACCA CTTGTCTTCC AGTCCCCATG CCCCGAAATT CCAAGGCTCT	4500
CATCCCTGAA ACCTAGGACT CAGGCTCTCC CTACCTCAGC CCCAGGAGTC TAAACCTTTA	4560
ACTTCTCTT TCCCTGGGAC TAAGGAGTGC TGCACCCCAG GCGCCTCCCT TACCCACAT	4620
CCCTCCTCAG CCTCCCCCTC TCAGCCTCAG TGCATTTGCT AATTCGCCTT TCCTCCCCTG	4680
CAGCCATGTG GCTCCGGAGC CATCGTCAGC TCTGCCTGGC CTTCCTGCTA GTCTGTGTCC	4740
TCTCTGTAAT CTTCTTCCTC CATATCCATC AAGACAGCTT TCCACATGGC CTAGGCCTGT	4800
CGATCCTGTG TCCAGACCGC CGCCTGGTGA CACCCCCAGT GGCCATCTTC TGCCTGCCGG	4860
GTACTGCGAT GGGCCCCAAC GCCTCCTCTT CCTGTCCCCA GCACCCTGCT TCCCTCTCCG	4920
GCACCTGGAC TGTCTACCCC AATGGCCGGT TTGGTAATCA GATGGGACAG TATGCCACGC	4980
TGCTGGCTCT GGGCCAGCTC AACGGCCGCC GGGCCTTTAT CCTGCCTGCC ATGCATGCCG	5040
CCCTGGCCCC GGTATTCCGC ATCACCCTGC CCGTGCTGGC CCCAGAAGTG GACAGCCGCA	5100
CGCCGTGGCG GGAGCTGCAG CTTACGACT GGATGTCGGA GGAGTACGCG GACTTGAGAG	5160
ATCCTTTCTT GAAGCTCTCT GGCTTCCCCT GCTCTTGGAC TTTCTTCCAC CATCTCCGGG	5220
AACAGATCCG CAGAGAGTTC ACCCTGCACG ACCACCTTCG GGAAGAGGCG CAGAGTGTGC	5280
TGGGTGAGCT CCGCCTGGGC CGCACAGGGG ACCGCCCGCG CACCTTTGTC GGCGTCCACG	5340
TGCGCCGTGG GGAATATCTG CAGGTTATGC CTCAGCGCTG GAAGGGTGTG GTGGGCGACA	5400
GCGCCTACCT CCGGCAGGCC ATGGACTGGT TCCGGGCACG GCACGAAGCC CCCGTTTTCG	5460

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TGGTCACCAG CAACGGCATG GAGTGGTGTA AAGAAAACAT CGACACCTCC CAGGGCGATG	5520
TGACGTTTGC TGGCGATGGA CAGGAGGCTA CACCGTGGAA AGACTTTGCC CTGCTCACAC	5580
AGTGCAACCA CACCATTATG ACCATTGGCA CCTTCGGCTT CTGGGCTGCC TACCTGGCTG	5640
GCGGAGACAC TGTCTACCTG GCCAACTTCA CCCTGCCAGA CTCTGAGTTC CTGAAGATCT	5700
TTAAGCCGGA GCGGGCCTTC CTGCCCCGAGT GGGTGGGCAT TAATGCAGAC TTGTCTCCAC	5760
TCTGGACATT GGCTAAGCCT TGAGAGCCAG GGAGACTTTC TGAAGTAGCC TGATCTTTCT	5820
AGAGCCAGCA GTACGTGGCT TCAGAGGCCT GGCATCTTCT GGAGAAGCTT GTGGTGTTC	5880
TGAAGCAAAT GGGTGCCCGT ATCCAGAGTG ATTCTAGTTG GGAGAGTTGG AGAGAAGGGG	5940
GACGTTTCTG GAACTGTCTG AATATTCTAG AACTAGCAAA ACATCTTTTC CTGATGGCTG	6000
GCAGGCAGTT CTAGAAGCCA CAGTGCCAC CTGCTCTTCC CAGCCCATAT CTACAGTACT	6060
TCCAGATGGC TGCCCCCAGG AATGGGGAAC TCTCCCTCTG GTCTACTCTA GAAGAGGGGT	6120
TACTTCTCCC CTGGGTCCTC CAAAGACTGA AGGAGCATAT GATTGCTCCA GAGCAAGCAT	6180
TCACCAAGTC CCCTTCTGTG TTTCTGGAGT GATTCTAGAG GGAGACTTGT TCTAGAGAGG	6240
ACCAGGTTTG ATGCCTGTGA AGAACCCTGC AGGGCCCTTA TGGACAGGAT GGGGTTCTGG	6300
AAATCCAGAT AACTAAGGTG AAGAATCTTT TTAGTTTTTT TTTTTTTTTT TTGGAGACAG	6360
GGTCTCGCTC TGTGCCCCAG GCTGGAGTGC AGTGGCGTGA TCTTGGCTCA CTGCAACTTC	6420
CGCCTCCTGT GTTCAAGCGA TTCTCCTGTC TCAGCCTCCT GAGTAGATGG GACTACAGGC	6480
ACAGGCCATT ATGCCTGGCT AATTTTTGTA TTTTGTAGTAG AGACAGGGTT TCACCATGTT	6540
GGCCGGGATG GTCTCGATCT CCTGACCTTG TCATCCACCT GTCTTGGCCT CCCAAAGTGC	6600
TGGGATTACT GGCATGAGCC ACTGTGCCCA GCCCGGATAT TTTTTTTTAA TTATTTATTT	6660
ATTTATTTAT TTATTGAGAC GGAGTCTTGC TCTGTAGCCC AGGCCAGAGT GCAGTGGCGC	6720
GATCTCAGCT CACTGCAAGC TCTGCCTCCC GGGTTCATGC CATTCTGCCT CAGCCTCCTG	6780
AGTAGCTGGG ACTACAGGCG CCCGCCACCA CGCCCGGCTA ATTTTTTTTG TATTTTGTAGT	6840
AGAGACGGGG TTTCATCGTG TTAACCAGGA TGGTCTCGAT CTCCTGACCT CGTGATCTGC	6900
CCACCTCGGC CTCCCACAGT GCTGGGATTA CCGGCGTGAG CCACCATGCC TGGCCCGGAT	6960
AATTTTTTTT AATTTTTGTA GAGACGAGGT CTTGTGATAT TGCCCAGGCT GTTCTTCAAC	7020

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TCCTGGGCTC AAGCAGTCCT CCCACCTTGG CCTCCCAGAA TGCTGGGTTT ATAGATGTGA	7080
GCCAGCACAC CGGGCCAAGT GAAGAATCTA ATGAATGTGC AACCTAATTG TAGCATCTAA	7140
TGAATGTTCC ACCATTGCTG GAAAAATTGA GATGGAAAAC AAACCATCTC TAGTTGGCCA	7200
GCGTCTTGCT CTGTTACAG TCTCTGGAAA AGCTGGGGTA GTTGGTGAGC AGAGCGGGAC	7260
TCTGTCCAAC AAGCCCCACA GCCCCTCAA GACTTTTTTTT TGTTTGTTTT GAGCAGACAG	7320
GCTAAAATGT GAACGTGGGG TGAGGGATCA CTGCCAAAAT GGTACAGCTT CTGGAGCAGA	7380
ACTTTCAGG GATCCAGGGA CACTTTTTTTT TAAAGCTCAT AAAGTCCAA GAGCTCCATA	7440
TATTGGGTGT GAGTTCAGGT TGCCTCTCAC AATGAAGGAA GTTGGTCTTT GTCTGCAGGT	7500
GGGCTGCTGA GGGTCTGGGA TCTGTTTTCT GGAAGTGTGC AGGTATAAAC ACACCCTCTG	7560
TGCTTGTGAC AAAGTGGCAG GTACCGTGCT CATTGCTAAC CACTGTCTGT CCCTGAACTC	7620
CCAGAACCAC TACATCTGGC TTTGGGCAGG TCTGAGATAA AACGATCTAA AGGTAGGCAG	7680
ACCCTGGACC CAGCCTCAGA TCCAGGCAGG AGCACGAGGT CTGGCCAAGG TGGACGGGGT	7740
TGTCGAGATC TCAGGAGCCC CTTGCTGTTT TTTGGAGGGT GAAAGAAGAA ACCTTAAACA	7800
TAGTCAGCTC TGATCACATC CCCTGTCTAC TCATCCAGAC CCCATGCCTG TAGGCTTATC	7860
AGGGAGTTAC AGTTACAATT GTTACAGTAC TGTTCCCAAC TCAGCTGCCA CGGGTGAGAG	7920
AGCAGGAGGT ATGAATTAAA AGTCTACAGC ACTAACCCGT GTCTCTGTAG CTTTTTTGGA	7980
GCCAGAGCCA CTGTGTATGT GTGTGTGGGT TTGTGTGTGT GTGTGTGTGT GTGTGTGTGT	8040
AAGAGAGTGG AGGAAAAGGT GGGGTACTTC TGAAGACTTT TATTTTTTTT TAATTAATTT	8100
ATTTTTTTTC AGAGATCGAG TCTTGCTCTG TGGCCCAGGC TGGAGTGCAG TAGTGTGATC	8160
TCGGCCCACT GCAA	8174

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 365 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Trp Leu Arg Ser His Arg Gln Leu Cys Leu Ala Phe Leu Leu Val  
 1 5 10 15  
 Cys Val Leu Ser Val Ile Phe Phe Leu His Ile His Gln Asp Ser Phe  
 20 25 30  
 Pro His Gly Leu Gly Leu Ser Ile Leu Cys Pro Asp Arg Arg Leu Val  
 35 40 45  
 Thr Pro Pro Val Ala Ile Phe Cys Leu Pro Gly Thr Ala Met Gly Pro  
 50 55 60  
 Asn Ala Ser Ser Ser Cys Pro Gln His Pro Ala Ser Leu Ser Gly Thr  
 65 70 75 80  
 Trp Thr Val Tyr Pro Asn Gly Arg Phe Gly Asn Gln Met Gly Gln Tyr  
 85 90 95  
 Ala Thr Leu Leu Ala Leu Ala Gln Leu Asn Gly Arg Arg Ala Phe Ile  
 100 105 110  
 Leu Pro Ala Met His Ala Ala Leu Ala Pro Val Phe Arg Ile Thr Leu  
 115 120 125  
 Pro Val Leu Ala Pro Glu Val Asp Ser Arg Thr Pro Trp Arg Glu Leu  
 130 135 140  
 Gln Leu His Asp Trp Met Ser Glu Glu Tyr Ala Asp Leu Arg Asp Pro  
 145 150 155 160  
 Phe Leu Lys Leu Ser Gly Phe Pro Cys Ser Trp Thr Phe Phe His His  
 165 170 175  
 Leu Arg Glu Gln Ile Arg Arg Glu Phe Thr Leu His Asp His Leu Arg  
 180 185 190  
 Glu Glu Ala Gln Ser Val Leu Gly Gln Leu Arg Leu Gly Arg Thr Gly  
 195 200 205  
 Asp Arg Pro Arg Thr Phe Val Gly Val His Val Arg Arg Gly Asp Tyr  
 210 215 220  
 Leu Gln Val Met Pro Gln Arg Trp Lys Gly Val Val Gly Asp Ser Ala  
 225 230 235 240  
 Tyr Leu Arg Gln Ala Met Asp Trp Phe Arg Ala Arg His Glu Ala Pro  
 245 250 255  
 Val Phe Val Val Thr Ser Asn Gly Met Glu Trp Cys Lys Glu Asn Ile

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	260		265		270
Asp Thr Ser Gln Gly Asp Val Thr Phe Ala Gly Asp Gly Gln Glu Ala					
275		280		285	
Thr Pro Trp Lys Asp Phe Ala Leu Leu Thr Gln Cys Asn His Thr Ile					
290		295		300	
Met Thr Ile Gly Thr Phe Gly Phe Trp Ala Ala Tyr Leu Ala Gly Gly					
305		310		315	320
Asp Thr Val Tyr Leu Ala Asn Phe Thr Leu Pro Asp Ser Glu Phe Leu					
	325		330		335
Lys Ile Phe Lys Pro Glu Ala Ala Phe Leu Pro Glu Trp Val Gly Ile					
	340		345		350
Asn Ala Asp Leu Ser Pro Leu Trp Thr Leu Ala Lys Pro					
	355		360		365

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3647 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGCAGAGAG CGCCACCCGG AAGCCACTTT TATAGAAGCT TTTACACACA ATGCTTGATT	60
TTTTTTTTTT TTTTCCGAGA CGGAGTCTCG CTTTGTGCGC CAGGCTGGAG TGCAGTGGCG	120
CGATCTGGGC TCACTGCAAG CTCCGCCTCC TGGGTTGACG CCATTCTCCT GCCTCAGCTT	180
CCCGAGTAGC TGGGACTACA GGCGCCCGCC ACCAAGCCTG GCTAATTTTT TTTTATTTTT	240
AGTGGAGACA GAGTTTCACC GTGTTAGCCA GGATGGTCTC GATCTCCTGA CCTCGGGATC	300
CGCCCGCCTC GGCCTCCCAA AGTGCTGGGA GTATAGGCGT GAGCCACCGC GCCTGGCCTA	360
TACTTGATTT TTAATGAAAA CATTCTTAAA TTCATATGGC TAACGCAAAT TTATTTTCTG	420

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TAGGCATAAC ATCAAAAACA CCTGGCAGGA CTGCCCCATT CCCAGCACTG TCTAGTTCTC	480
CCCTAGTATC AGTGGGACTC CACTGATGCA CAGCTGTGAT CTAATAAAAC TTCTCTCAAA	540
ACTTTCTCCT CTCCTTAGGT CAGCAGCCCC GCCCCTGATC TATTTGGAAA TCCCCTGAAT	600
AAAAGTTGAA TATCATAAAC CAAAGCGAAC ACCCAGAAAT TCAAATTCAA CCCGTAGGTA	660
AAAAATTTCT CAAGTGAAGT TAGACGTAGA TGTCTCCAGT GTCGCCTAAT AAGGTAGAAG	720
AGGCCAGTGC GATACTGTCT TTACACCCTT AACTTGGGTG CTAGAATATT TATCTTCGTC	780
ATCATTTTAT CATCCAACT ATTTTGCATA ACTTTCATGG GTGCAGAAAA TGTTTTTTAA	840
GTGCTTGGTA AAATTAATAG TGATATTCAT TCATTCATCT CACTGAACAG GCAATAAATT	900
CCTTGACGAC AAGGGCCTTG GGGGGGGCCA CATCTTCATC TTTGGTTTAT GAGTCCTGTG	960
CGTCTTGGTA CAAGCAATAC TACTATGAGC CGGCAAGTCA GACTTATTTG GTAGGGGACC	1020
AAAGGAAAGA ACATGTTTTG ATTGCTAAGA AAACATTTTG TTCTCTATCC TTTACTGGGC	1080
TGGCAGGCAA AGGAAATGTT CTTATGAGCA CTCACATTGA AAACTTAAGT TCTTCACCAA	1140
ATGCAGAGAC TCTGAAGGCC ACGCCGCTGC GGGCTGCCTC CACAATTCGA CCGTCTCGGC	1200
GGGCCACGAG ATCCTGGCCA CGGATGCGGT GGCCGCGCCT CTGCTCGCAC GTTCCCCCGG	1260
CCTCTGGAAT CCCTCCCTCC CTCAATCCCT CCCTCCGGCG GGCCTCGCTG GCGGGTGGCT	1320
AGGCCCAACG GCAGGAAGCC GACGCTATCC TCCGTTCCGC GGCGCCGGGT CCGCCTTCCG	1380
TCTGTTCTAG GGCCTGCTCC TGCGCGGCAG CTGCTTTAGA AGGTCTCGAG CCTCCTGTAC	1440
CTTCCCAGGG ATGAACCGGG CCTTCCCTCT GGAAGGCGAG GGTTCGGGCC ACAGTGAGCG	1500
AGGGCCAGGG CCGTGGGCGC GCGCAGAGGG AAACCGGATC AGTTGAGAGA GAATCAAGAG	1560
TAGCGGATGA GGCCTTGTG GGGCGCGGCC CGGAAGCCCT CGGGCGCGGG CTGGGAGAAG	1620
GAGTGGGCGG AGGCGCCGCA GGAGGCTCCC GGGGCTTGGT CGGGCCGGCT GGGCCCCGGG	1680
CGCAGTGGA GAAAGGGACG GGCGGTGCCC GGTGGGCGT CCTGGCCAGC TCACCTTGCC	1740
CTGGCGGCTC GCCCCGCCCC GCACTTGGGA GGAGCAGGGC AGGGCCCCCG GCCTTTGCAT	1800
TCTGGGACCG CCCCCTTCCA TTCCCGGGCC AGCGGCGAGC GGCAGCGACG GCTGGAGCCG	1860
CAGCTACAGC ATGAGAGCCG GTGCCGCTCC TCCACGCCTG CGGACGCGTG GCGAGCGGAG	1920
GCAGCGCTGC CTGTTGCGCG CATGGGGGCA CCGTGGGGCT CGCCGACGGC GGCGGCGGGC	1980



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GGGCGGCGCG GGTGGCGCCG AGGCCGGGGG CTGCCATGGA CCGTCTGTGT GCTGGCGGCC 2040  
GCCGGCTTGA CGTGACGGC GCTGATCACC TACGCTTGCT GGGGGCAGCT GCCGCCGCTG 2100  
CCCTGGGCGT CGCCAACCCC GTCGCGACCG GTGGGCGTGC TGCTGTGGTG GGAGCCCTTC 2160  
GGGGGGCGCG ATAGCGCCCC GAGGCCGCCC CCTGACTGCC CGCTGCGCTT CAACATCAGC 2220  
GGCTGCCGCC TGCTACCGA CCGCGCGTCC TACGGAGAGG CTCAGGCCGT GCTTTTCCAC 2280  
CACCGCGACC TCGTGAAGGG GCCCCCGAC TGGCCCCCGC CCTGGGGCAT CCAGGCGCAC 2340  
ACTGCCGAGG AGGTGGATCT GCGCGTGTG GACTACGAGG AGGCAGCGGC GCGGCAGAA 2400  
GCCCTGGCGA CCTCCAGCCC CAGGCCCCCG GGCCAGCGCT GGGTTTGGAT GAACTTCGAG 2460  
TCGCCCTCGC ACTCCCCGGG GCTGCGAAGC CTGGCAAGTA ACCTCTTCAA CTGGACGCTC 2520  
TCCTACCGGG CGGACTCGGA CGTCTTTGTG CCTTATGGCT ACCTCTACCC CAGAAGCCAC 2580  
CCCGGCGACC CGCCCTCAGG CCTGGCCCCG CCACTGTCCA GGAAACAGGG GCTGGTGGCA 2640  
TGGGTGGTGA GCCACTGGGA CGACCGCCAG GCCCGGGTCC GCTACTACCA CCAACTGAGC 2700  
CAACATGTGA CCGTGGACGT GTTCGGCCGG GCGGGGCCGG GGCAGCCGGT GCCCGAAATT 2760  
GGGCTCCTGC ACACAGTGGC CCGCTACAAG TTCTACCTGG CTTTCGAGAA CTCGCAGCAC 2820  
CTGGATTATA TCACCGAGAA GCTCTGGCGC AACGCGTTGC TCGCTGGGGC GGTGCCGGTG 2880  
GTGCTGGGCC CAGACCGTGC CAACTACGAG GCGTTTGTGC CCCGCGGCGC CTTTCATCCAC 2940  
GTGGACGACT TCCCAAGTGC CTCCTCCCTG GCCTCGTACC TGCTTTTCCT CGACCGCAAC 3000  
CCCGCGGTCT ATCGCCGCTA CTTCCACTGG CGCCGGAGCT ACGCTGTCCA CATCACCTCC 3060  
TTCTGGGACG AGCCTTGGTG CCGGGTGTGC CAGGCTGTAC AGAGGGCTGG GGACCGGCCC 3120  
AAGAGCATAC GGAAGTTGGC CAGCTGGTTC GAGCGGTGAA GCCGCGCTCC CCTGGAAGCG 3180  
ACCCAGGGGA GCCCAAGTTG TCAGCTTTTT GATCCTCTAC TGTGCATCTC CTTGACTGCC 3240  
GCATCATGGG AGTAAGTTCT TCAAACACCC ATTTTGTGCTC TATGGGAAAA AAACGATTTA 3300  
CCAATTAATA TTAATCAGCA CAGAGATGGG GGCCCGGTTT CCATATTTTT TGCACAGCTA 3360  
GCAATTGGGC TCCCTTTGCT GCTGATGGGC ATCATTTGTT AGGGGTGAAG GAGGGGGTTC 3420  
TTCCTCACCT TGTAACCAAGT GCAGAAATGA AATAGCTTAG CGGCAAGAAG CCGTTGAGGC 3480  
GGTTTCCTGA ATTTCCCAT CTGCCACAGG CCATATTTGT GGCCCGTGCA GCTTCCAAAT 3540

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CTCATACACA ACTGTTCCCG ATTACGTTT TTCTGGACCA AGGTGAAGCA AATTTGTGGT 3600  
 TGTAGAAGGA GCCTTGTTGG TGGAGAGTGG AAGGACTGTG GCTGCAG 3647

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 405 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gly Ala Pro Trp Gly Ser Pro Thr Ala Ala Ala Gly Gly Arg Arg  
 1 5 10 15  
 Gly Trp Arg Arg Gly Arg Gly Leu Pro Trp Thr Val Cys Val Leu Ala  
 20 25 30  
 Ala Ala Gly Leu Thr Cys Thr Ala Leu Ile Thr Tyr Ala Cys Trp Gly  
 35 40 45  
 Gln Leu Pro Pro Leu Pro Trp Ala Ser Pro Thr Pro Ser Arg Pro Val  
 50 55 60  
 Gly Val Leu Leu Trp Trp Glu Pro Phe Gly Gly Arg Asp Ser Ala Pro  
 65 70 75 80  
 Arg Pro Pro Pro Asp Cys Pro Leu Arg Phe Asn Ile Ser Gly Cys Arg  
 85 90 95  
 Leu Leu Thr Asp Arg Ala Ser Tyr Gly Glu Ala Gln Ala Val Leu Phe  
 100 105 110  
 His His Arg Asp Leu Val Lys Gly Pro Pro Asp Trp Pro Pro Pro Trp  
 115 120 125  
 Gly Ile Gln Ala His Thr Ala Glu Glu Val Asp Leu Arg Val Leu Asp  
 130 135 140  
 Tyr Glu Glu Ala Ala Ala Ala Glu Ala Leu Ala Thr Ser Ser Pro  
 145 150 155 160  
 Arg Pro Pro Gly Gln Arg Trp Val Trp Met Asn Phe Glu Ser Pro Ser  
 165 170 175

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His Ser Pro Gly Leu Arg Ser Leu Ala Ser Asn Leu Phe Asn Trp Thr  
 180 185 190  
 Leu Ser Tyr Arg Ala Asp Ser Asp Val Phe Val Pro Tyr Gly Tyr Leu  
 195 200 205  
 Tyr Pro Arg Ser His Pro Gly Asp Pro Pro Ser Gly Leu Ala Pro Pro  
 210 215 220  
 Leu Ser Arg Lys Gln Gly Leu Val Ala Trp Val Val Ser His Trp Asp  
 225 230 235 240  
 Glu Arg Gln Ala Arg Val Arg Tyr Tyr His Gln Leu Ser Gln His Val  
 245 250 255  
 Thr Val Asp Val Phe Gly Arg Gly Gly Pro Gly Gln Pro Val Pro Glu  
 260 265 270  
 Ile Gly Leu Leu His Thr Val Ala Arg Tyr Lys Phe Tyr Leu Ala Phe  
 275 280 285  
 Glu Asn Ser Gln His Leu Asp Tyr Ile Thr Glu Lys Leu Trp Arg Asn  
 290 295 300  
 Ala Leu Leu Ala Gly Ala Val Pro Val Val Leu Gly Pro Asp Arg Ala  
 305 310 315 320  
 Asn Tyr Glu Arg Phe Val Pro Arg Gly Ala Phe Ile His Val Asp Asp  
 325 330 335  
 Phe Pro Ser Ala Ser Ser Leu Ala Ser Tyr Leu Leu Phe Leu Asp Arg  
 340 345 350  
 Asn Pro Ala Val Tyr Arg Arg Tyr Phe His Trp Arg Arg Ser Tyr Ala  
 355 360 365  
 Val His Ile Thr Ser Phe Trp Asp Glu Pro Trp Cys Arg Val Cys Gln  
 370 375 380  
 Ala Val Gln Arg Ala Gly Asp Arg Pro Lys Ser Ile Arg Asn Leu Ala  
 385 390 395 400  
 Ser Trp Phe Glu Arg  
 405

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1488 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown

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(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGGGGGCAC CGTGGGGCTC GCCGACGGCG GCGGCGGGCG GGCGGCGCGG GTGGCGCCGA	60
GGCCCGGGGC TGCCATGGAC CGTCTGTGTG CTGGCGGGCCG CCGGCTTGAC GTGTACGGCG	120
CTGATCACCT ACGCTTGCTG GGGGCAGCTG CCGCCGCTGC CCTGGGCGTC GCCAACCCCG	180
TCGCGACCGG TGGGCGTGCT GCTGTGGTGG GAGCCCTTCG GGGGGCGCGA TAGCGCCCCG	240
AGGCCGCCCC CTGACTGCTG CTGGGGGCGAG CTGCCGCCGC TGCCCTGGGC GTCGCCAACC	300
CCGTCCCGAC CGGTGGGCGT GCTGCTGTGG TGGGAGCCCT TCGGGGGGCG CGATAGCGCC	360
CCGAGGCCGC CCCCTGACTG CCCGCTGCGC TTCAACATCA GCGGCTGCCG CCTGCTCACC	420
GACCGCGCGT CCTACGGAGA GGCTCAGGCC GTGCTTTTCC ACCACCGCGA CCTCGTGAAG	480
GGGCCCCCGG ACTGGCCCCC GCCCTGGGGC ATCCAGGCGC ACACTGCCGA GCCGCTGCGC	540
TTCAACATCA GCGGCTGCCG CCTGCTCACC GACCGCGCGT CCTACGGAGA GGCTCAGGCC	600
GTGCTTTTCC ACCACCGCGA CCTCGTGAAG GGGCCCCCGG ACTGGCCCCC GCCCTGGGGC	660
ATCCAGGCGC ACACTGCCGA GGAGGTGGAT CTGCGCGTGT TGGACTACGA GGAGGCAGCG	720
GCGGCGGCAG AAGCCCTGGC GACCTCCAGC CCCAGGCCCC CGGGCCAGCG CTGGGTTTGG	780
ATGAACTTCG AGTCGCCCTC GCACTCCCCG GGGCTGCGAA GCCTGGCAAG TAACCTCTTC	840
AACTGGACGC TCTCCTACCG GGC GGACTCG GACGTCTTTG TGCCTTATGG CTACCTCTAC	900
CCCAGAAGCC ACCCCGGCGA CCCGCCCTCA GGCCTGGCCC CGCCACTGTC CAGGAAACAG	960
GGGCTGGTGG CATGGGTGGT GAGCCACTGG GACGAGCGCC AGGCCCGGGT CCGCTACTAC	1020
CACCAACTGA GCCAACATGT GACCGTGGAC GTGTTTCGGC GGGGCGGGCC GGGGCAGCCG	1080
GTGCCCCGAA TTGGGCTCCT GCACACAGTG GCCCGCTACA AGTTCTACCT GGCTTTTCGAG	1140
AACTCGCAGC ACCTGGATTA TATCACCAGG AAGCTCTGGC GCAACGCGTT GCTCGCTGGG	1200
GCGGTGCCGG TGGTGTGGG CCCAGACCGT GCCAACTACG AGCGCTTTGT GCCCCGCGGC	1260
GCCTTCATCC ACGTGGACGA CTTCCCAAGT GCCTCCTCCC TGGCCTCGTA CCTGCTTTTC	1320

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CTCGACCGCA ACCCCGCGGT CTATCGCCGC TACTTCCACT GCGCCGGAG CTACGCTGTC 1380  
CACATCACCT CCTTCTGGGA CGAGCCTTGG TGCCGGGTGT GCCAGGCTGT ACAGAGGGCT 1440  
GGGGACCGGC CCAAGAGCAT ACGGAACTTG GCCAGCTGGT TCGAGCGG 1488

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1316 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTATGACAA GCTGTGTCAT AAATTATAAC AGCTTCTCTC AGGACACTGT GGCCAGGAAG 60  
TGGGTGATCT TCCTTAATGA CCCTCACTCC TCTCTCCTCT CTTCCCAGCT ACTCTGACCC 120  
ATGGATCCCC TGGGCCCAGC CAAGCCACAG TGGCTGTGGC GCCGCTGTCT GGCCGGGCTG 180  
CTGTTTCAGC TGCTGGTGGC TGTGTGTTTC TTCTCCTACC TGCGTGTGTC CCGAGACGAT 240  
GCCACTGGAT CCCCTAGGCC AGGGCTTATG GCAGTGGAAC CTGTCACCGG GGCTCCCAAT 300  
GGGTCCCGCT GCCAGGACAG CATGGCGACC CCTGCCCCACC CCACCCTACT GATCCTGCTG 360  
TGGACGTGGC CTTTAAACAC ACCCGTGGCT CTGCCCCGCT GCTCAGAGAT GGTGCCCCGGC 420  
GCGGCCGACT GCAACATCAC TGCCGACTCC AGTGTGTACC CACAGGCAGA CGCGGTCATC 480  
GTGCACCACT GGGATATCAT GTACAACCCC AGTGCCAACC TCCCGCCCCC CACCAGGCCG 540  
CAGGGGCAGC GCTGGATCTG GTTCAGCATG GAGTCCCCCA GCAACTGCCG GCACCTGGAA 600  
GCCCTGGACG GATACTTCAA TCTCACCATG TCCTACCGCA GCGACTCCGA CATCTTCACG 660  
CCCTACGGCT GGCTGGAGCC GTGGTCCGGC CAGCCTGCCC ACCCACCCTG CAACCTCTCG 720  
GCCAAGACCG AGCTGGTGGC CTGGGCGGTG TCCAAGTGA AGCCGGACTC GGCCAGGGTG 780  
CGCTACTACC AGAGCCTGCA GGCTCATCTC AAGGTGGACG TGTACGGACG CTCCACAAG 840  
CCCCTGCCCA AGGGGACCAT GATGGAGACG CTGTCCCGGT ACAAGTTCTA TCTGGCCTTC 900

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GAGAACTCCT TGCACCCCGA CTACATCACC GAGAAGCTGT GGAGGAACGC CCTGGAGGCC 960  
 TGGGCCGTGC CCGTGGTGCT GGGCCCCAGC AGAAGCAACT ACGAGAGGTT CCTGCCGCCC 1020  
 GACGCCTTCA TCCACGTGGA TGACTTCCAG AGCCCCAAGG ACCTGGCCCCG GTACCTGCAG 1080  
 GAGCTGGACA AGGACCACGC CCGCTACCTG AGCTACTTTC GCTGGCGGGA GACGCTGCGG 1140  
 CCTCGCTCCT TCAGCTGGGC ACTGGCTTTC TGCAAGGCCT GCTGGAAGCT GCAGCAGGAA 1200  
 TCCAGGTACC AGACGGTGCG CAGCATAGCG GCTTGGTTCA CCTGAGAGGC CGGCATGGGG 1260  
 CCTGGGCTGC CAGGGACCTC ACTTTCCAG GGCCTCACCT ACCTAGGGTC TCTAGA 1316

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 374 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Asp Pro Leu Gly Pro Ala Lys Pro Gln Trp Leu Trp Arg Arg Cys  
 1 5 10 15  
 Leu Ala Gly Leu Leu Phe Gln Leu Leu Val Ala Val Cys Phe Phe Ser  
 20 25 30  
 Tyr Leu Arg Val Ser Arg Asp Asp Ala Thr Gly Ser Pro Arg Pro Gly  
 35 40 45  
 Leu Met Ala Val Glu Pro Val Thr Gly Ala Pro Asn Gly Ser Arg Cys  
 50 55 60  
 Gln Asp Ser Met Ala Thr Pro Ala His Pro Thr Leu Leu Ile Leu Leu  
 65 70 75 80  
 Trp Thr Trp Pro Phe Asn Thr Pro Val Ala Leu Pro Arg Cys Ser Glu  
 85 90 95  
 Met Val Pro Gly Ala Ala Asp Cys Asn Ile Thr Ala Asp Ser Ser Val  
 100 105 110  
 Tyr Pro Gln Ala Asp Ala Val Ile Val His His Trp Asp Ile Met Tyr  
 115 120 125

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Asn Pro Ser Ala Asn Leu Pro Pro Pro Thr Arg Pro Gln Gly Gln Arg  
 130 135 140  
 Trp Ile Trp Phe Ser Met Glu Ser Pro Ser Asn Cys Arg His Leu Glu  
 145 150 155 160  
 Ala Leu Asp Gly Tyr Phe Asn Leu Thr Met Ser Tyr Arg Ser Asp Ser  
 165 170 175  
 Asp Ile Phe Thr Pro Tyr Gly Trp Leu Glu Pro Trp Ser Gly Gln Pro  
 180 185 190  
 Ala His Pro Pro Leu Asn Leu Ser Ala Lys Thr Glu Leu Val Ala Trp  
 195 200 205  
 Ala Val Ser Asn Trp Lys Pro Asp Ser Ala Arg Val Arg Tyr Tyr Gln  
 210 215 220  
 Ser Leu Gln Ala His Leu Lys Val Asp Val Tyr Gly Arg Ser His Lys  
 225 230 235 240  
 Pro Leu Pro Lys Gly Thr Met Met Glu Thr Leu Ser Arg Tyr Lys Phe  
 245 250 255  
 Tyr Leu Ala Phe Glu Asn Ser Leu His Pro Asp Tyr Ile Thr Glu Lys  
 260 265 270  
 Leu Trp Arg Asn Ala Leu Glu Ala Trp Ala Val Pro Val Val Leu Gly  
 275 280 285  
 Pro Ser Arg Ser Asn Tyr Glu Arg Phe Leu Pro Pro Asp Ala Phe Ile  
 290 295 300  
 His Val Asp Asp Phe Gln Ser Pro Lys Asp Leu Ala Arg Tyr Leu Gln  
 305 310 315 320  
 Glu Leu Asp Lys Asp His Ala Arg Tyr Leu Ser Tyr Phe Arg Trp Arg  
 325 330 335  
 Glu Thr Leu Arg Pro Arg Ser Phe Ser Trp Ala Leu Ala Phe Cys Lys  
 340 345 350  
 Ala Cys Trp Lys Leu Gln Gln Glu Ser Arg Tyr Gln Thr Val Arg Ser  
 355 360 365  
 Ile Ala Ala Trp Phe Thr  
 370

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 1086 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGGATCCCC TGGGTGCAGC CAAGCCACAA TGGCCATGGC GCCGCTGTCT GGCCGCACTG	60
CTATTTTCAGC TGCTGGTGGC TGTGTGTTTC TTCTCCTACC TGCCTGTGTC CCGAGACGAT	120
GCCACTGGAT CCCCTAGGGC TCCCAGTGGG TCCTCCCGAC AGGACACCAC TCCCACCCGC	180
CCCACCCTCC TGATCCTGCT ATGGACATGG CCTTTCACA TCCCTGTGGC TCTGTCCCGC	240
TGTTTCAGAGA TGGTGCCCGG CACAGCCGAC TGCCACATCA CTGCCGACCG CAAGGTGTAC	300
CCACAGGCAG ACACGGTCAT CGTGCACCAC TGGGATATCA TGTCCAACCC TAAGTCACGC	360
CTCCACCTT CCCCAGGGCC GCAGGGGCAG CGCTGGATCT GGTCAACTT GGAGCCACCC	420
CCTAACTGCC AGCACCTGGA AGCCCTGGAC AGATACTTCA ATCTCACCAT GTCCTACCGC	480
AGCGACTCCG ACATCTTCAC GCCCTACGGC TGGCTGGAGC CGTGGTCCGG CCAGCCTGCC	540
CACCCACCGC TCAACCTCTC GGCCAAGACC GAGCTGGTGG CCTGGGCGGT GTCCAAGTGG	600
AAGCCGGACT CAGCCAGGGT GCGCTACTAC CAGAGCCTGC AGGCTCATCT CAAGGTGGAC	660
GTGTACGGAC GCTCCACAA GCCCTGCCC AAGGGGACCA TGATGGAGAC GCTGTCCCGG	720
TACAAGTTCT ACCTGGCCTT CGAGAACTCC TTGCACCCCG ACTACATCAC CGAGAAGCTG	780
TGGAGGAACG CCCTGGAGGC CTGGGCCGTG CCCGTGGTGC TGGGCCCCAG CAGAAGCAAC	840
TACGAGAGGT TCCTGCCACC CGACGCCTTC ATCCACGTGG ACGACTTCCA GAGCCCCAAG	900
GACCTGGCCC GGTACCTGCA GGAGCTGGAC AAGGACCACG CCCGCTACCT GAGCTACTTT	960
CGCTGGCGGG AGACGCTGCG GCCTCGCTCC TTCAGCTGGG CACTGGATTT CTGCAAGGCC	1020
TGCTGGAAAC TGCAGCAGGA ATCCAGGTAC CAGACGGTGC GCAGCATAGC GGCTTGGTTC	1080
ACCTGA	1086

(2) INFORMATION FOR SEQ ID NO:13:



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## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1654 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTTTCTCATC TGTGAAACAG GAATAATAAC AGCTCTTCTC AGGACTCATG GCCTGGAGCT	60
TTGGTAAGCA GGAGATTGTC ATCAATGACC CTCACTCCTC TCTCCCCACT TCCCAGAGAC	120
TCTGACCCAT GGATCCCCTG GGCCCGGCCA AGCCACAGTG GTCGTGGCGC TGCTGTCTGA	180
CCACGCTGCT GTTTCAGCTG CTGATGGCTG TGTGTTTCTT CTCCTATCTG CGTGTGTCTC	240
AAGACGATCC CACTGTGTAC CCTAATGGGT CCCGCTTCCC AGACAGCACA GGGACCCCCG	300
CCCACTCCAT CCCCCTGATC CTGCTGTGGA CGTGGCCTTT TAACAAACCC ATAGCTCTGC	360
CCCGCTGCTC AGAGATGGTG CCTGGCACGG CTGACTGCAA CATCACTGCC GACCGCAAGG	420
TGTATCCACA GGCAGACGCG GTCATCGTGC ACCACCGAGA GGTCATGTAC AACCCCAGTG	480
CCCAGCTCCC ACGCTCCCCG AGGCGGCAGG GGCAGCGATG GATCTGGTTC AGCATGGAGT	540
CCCCAAGCCA CTGCTGGCAG CTGAAAGCCA TGGACGGATA CTTCAATCTC ACCATGTCCT	600
ACCGCAGCGA CTCCGACATC TTCACGCCCT ACGGCTGGCT GGAGCCGTGG TCCGGCCAGC	660
CTGCCCACCC ACCGCTCAAC CTCTCGGCCA AGACCGAGCT GGTGGCCTGG GCAGTGTCCT	720
ACTGGGGGCC AAACCTCCGCC AGGGTGCGCT ACTACCAGAG CCTGCAGGCC CATCTCAAGG	780
TGGACGTGTA CGGACGCTCC CACAAGCCCC TGCCCCAGGG AACCATGATG GAGACGCTGT	840
CCCGGTACAA GTTCTATCTG GCCTTCGAGA ACTCCTTGCA CCCCAGTAC ATCACCGAGA	900
AGCTGTGGAG GAACGCCCTG GAGGCCTGGG CCGTGCCCGT GGTGCTGGGC CCCAGCAGAA	960
GCAACTACGA GAGGTTCTCTG CCACCCGACG CCTTCATCCA CGTGGACGAC TTCCAGAGCC	1020
CCAAGGACCT GGCCCGGTAC CTGCAGGAGC TGGACAAGGA CCACGCCCCG TACCTGAGCT	1080
ACTTTCGCTG GCGGGAGACG CTGCGGCCTC GCTCCTTCAG CTGGGCACTC GCTTTCTGCA	1140
AGGCCTGCTG GAAACTGCAG GAGGAATCCA GGTACCAGAC ACGCGGCATA GCGGCTTGGT	1200

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TCACCTGAGA GGCTGGTGTG GGGCCTGGGC TGCCAGGAAC CTCATTTTCC TGGGGCCTCA 1260  
 CCTGAGTGGG GGCCTCATCT ACCTAAGGAC TCGTTTGCCT GAAGCTTCAC CTGCCTGAGG 1320  
 ACTCACCTGC CTGGGACGGT CACCTGTTGC AGCTTCACCT GCCTGGGGAT TCACCTACCT 1380  
 GGGTCCTCAC TTTCCTGGGG CCTCACCTGC TGGAGTCTTC GGTGGCCAGG TATGTCCCTT 1440  
 ACCTGGGATT TCACATGCTG GCTTCCAGGA GCGTCCCCTG CGGAAGCCTG GCCTGCTGGG 1500  
 GATGTCTCCT GGGGACTTTG CCTACTGGGG ACCTCGGCTG TTGGGGACTT TACCTGCTGG 1560  
 GACCTGCTCC CAGAGACCTT CCACACTGAA TCTCACCTGC TAGGAGCCTC ACCTGCTGGG 1620  
 GACCTCACCC TGGAGGCACT GGGCCCTGGG AACT 1654

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 359 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Asp Pro Leu Gly Pro Ala Lys Pro Gln Trp Ser Trp Arg Cys Cys  
 1 5 10 15  
 Leu Thr Thr Leu Leu Phe Gln Leu Leu Met Ala Val Cys Phe Phe Ser  
 20 25 30  
 Tyr Leu Arg Val Ser Gln Asp Asp Pro Thr Val Tyr Pro Asn Gly Ser  
 35 40 45  
 Arg Phe Pro Asp Ser Thr Gly Thr Pro Ala His Ser Ile Pro Leu Ile  
 50 55 60  
 Leu Leu Trp Thr Trp Pro Phe Asn Lys Pro Ile Ala Leu Pro Arg Cys  
 65 70 75 80  
 Ser Glu Met Val Pro Gly Thr Ala Asp Cys Asn Ile Thr Ala Asp Arg  
 85 90 95  
 Lys Val Tyr Pro Gln Ala Asp Ala Val Ile Val His His Arg Glu Val  
 100 105 110  
 Met Tyr Asn Pro Ser Ala Gln Leu Pro Arg Ser Pro Arg Arg Gln Gly

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115					120					125					
Gln	Arg	Trp	Ile	Trp	Phe	Ser	Met	Glu	Ser	Pro	Ser	His	Cys	Trp	Gln
130						135					140				
Leu	Lys	Ala	Met	Asp	Gly	Tyr	Phe	Asn	Leu	Thr	Met	Ser	Tyr	Arg	Ser
145					150					155					160
Asp	Ser	Asp	Ile	Phe	Thr	Pro	Tyr	Gly	Trp	Leu	Glu	Pro	Trp	Ser	Gly
				165					170					175	
Gln	Pro	Ala	His	Pro	Pro	Leu	Asn	Leu	Ser	Ala	Lys	Thr	Glu	Leu	Val
			180					185					190		
Ala	Trp	Ala	Val	Ser	Asn	Trp	Gly	Pro	Asn	Ser	Ala	Arg	Val	Arg	Tyr
		195					200					205			
Tyr	Gln	Ser	Leu	Gln	Ala	His	Leu	Lys	Val	Asp	Val	Tyr	Gly	Arg	Ser
210						215					220				
His	Lys	Pro	Leu	Pro	Gln	Gly	Thr	Met	Met	Glu	Thr	Leu	Ser	Arg	Tyr
225						230					235				240
Lys	Phe	Tyr	Leu	Ala	Phe	Glu	Asn	Ser	Leu	His	Pro	Asp	Tyr	Ile	Thr
				245					250					255	
Glu	Lys	Leu	Trp	Arg	Asn	Ala	Leu	Glu	Ala	Trp	Ala	Val	Pro	Val	Val
			260					265					270		
Leu	Gly	Pro	Ser	Arg	Ser	Asn	Tyr	Glu	Arg	Phe	Leu	Pro	Pro	Asp	Ala
		275					280					285			
Phe	Ile	His	Val	Asp	Asp	Phe	Gln	Ser	Pro	Lys	Asp	Leu	Ala	Arg	Tyr
290						295					300				
Leu	Gln	Glu	Leu	Asp	Lys	Asp	His	Ala	Arg	Tyr	Leu	Ser	Tyr	Phe	Arg
305						310					315				320
Trp	Arg	Glu	Thr	Leu	Arg	Pro	Arg	Ser	Phe	Ser	Trp	Ala	Leu	Ala	Phe
				325					330					335	
Cys	Lys	Ala	Cys	Trp	Lys	Leu	Gln	Glu	Glu	Ser	Arg	Tyr	Gln	Thr	Arg
			340					345					350		
Gly	Ile	Ala	Ala	Trp	Phe	Thr									
			355												

WHAT IS CLAIMED AS NEW AND DESIRED TO BE SECURED BY  
LETTERS PATENT OF THE UNITED STATES IS:

1. A polypeptide having an amino acid sequence selected from the group consisting of:

- (a) a first subsequence corresponding to amino acid positions 1 to 301 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to amino acid positions 301 to 359 of Fuc-TVI;
- (b) a first subsequence corresponding to amino acid positions 1 to 160 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to amino acid positions 160 to 359 of Fuc-TVI;
- (c) a first subsequence corresponding to amino acid positions 1 to 300 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to amino acid positions 302 to 361 of Fuc-TIII;
- (d) a first subsequence corresponding to amino acid positions 1 to 159 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to amino acid positions 161 to 361 of Fuc-TIII;
- (e) a first subsequence corresponding to amino acid positions 1 to 33 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 34 to 69 of Fuc-TVI connected to the amino

terminus of a third subsequence corresponding to amino acid positions 71 to 361 of Fuc-TIII;

- (f) a first subsequence corresponding to amino acid positions 1 to 70 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 70 to 85 of Fuc-TVI connected to the amino terminus of a third subsequence corresponding to amino acid positions 87 to 361 of Fuc-TIII;
- (g) a first subsequence corresponding to amino acid positions 1 to 86 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 86 to 98 of Fuc-TVI connected to the amino terminus of a third subsequence corresponding to amino acid positions 100 to 361 of Fuc-TIII;
- (h) a first subsequence corresponding to amino acid positions 1 to 99 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 99 to 129 of Fuc-TVI connected to the amino terminus of a third subsequence corresponding to amino acid positions 131 to 361 of Fuc-TIII;
- (i) a first subsequence corresponding to amino acid positions 1 to 130 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 130 to 159 of Fuc-TVI connected to the amino terminus of a third subsequence corresponding to amino acid positions 161 to 361 of Fuc-TIII;

- (j) a first subsequence corresponding to amino acid positions 1 to 33 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to positions 34 to 70 of Fuc-TIII connected to the amino terminus of a third subsequence corresponding to amino acid positions 70 to 359 of Fuc-TVI;
- (k) a first subsequence corresponding to amino acid positions 1 to 69 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to positions 71 to 86 of Fuc-TIII connected to the amino terminus of a third subsequence corresponding to amino acid positions 86 to 359 of Fuc-TVI;
- (l) a first subsequence corresponding to amino acid positions 1 to 85 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to positions 87 to 99 of Fuc-TIII connected to the amino terminus of a third subsequence corresponding to amino acid positions 99 to 359 of Fuc-TVI;
- (m) a first subsequence corresponding to amino acid positions 1 to 98 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to positions 100 to 130 of Fuc-TIII connected to the amino terminus of a third subsequence corresponding to amino acid positions 130 to 359 of Fuc-TVI;
- (n) a first subsequence corresponding to amino acid positions 1 to 129 of Fuc-TVI connected to the amino

terminus of a second subsequence corresponding to positions 131 to 160 of Fuc-TIII connected to the amino terminus of a third subsequence corresponding to amino acid positions 160 to 359 of Fuc-TVI;

- (o) a first subsequence corresponding to amino acid positions 1 to 33 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 34 to 98 of Fuc-TVI connected to the amino terminus of a third subsequence corresponding to amino acid positions 100 to 361 of Fuc-TIII;
- (p) a first subsequence corresponding to amino acid positions 1 to 86 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 86 to 159 of Fuc-TVI connected to the amino terminus of a third subsequence corresponding to amino acid positions 161 to 361 of Fuc-TIII;
- (q) a first subsequence corresponding to amino acid positions 1 to 33 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to positions 34 to 99 of Fuc-TIII connected to the amino terminus of a third subsequence corresponding to amino acid positions 99 to 359 of Fuc-TVI;
- (r) a first subsequence corresponding to amino acid positions 1 to 85 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to positions 87 to 160 of Fuc-TIII connected to the

amino terminus of a third subsequence corresponding to amino acid positions 160 to 359 of Fuc-TVI;

- (s) a first subsequence corresponding to amino acid positions 1 to 99 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 99 to 159 of Fuc-TVI connected to the amino terminus of a third subsequence corresponding to amino acid positions 161 to 361 of Fuc-TIII;
- (t) a first subsequence corresponding to amino acid positions 1 to 98 of Fuc-TVI connected to the amino terminus of a second subsequence corresponding to positions 100 to 160 of Fuc-TIII connected to the amino terminus of a third subsequence corresponding to amino acid positions 160 to 359 of Fuc-TVI;
- (u) a first subsequence corresponding to amino acid positions 1 to 99 of Fuc-TIII connected to the amino terminus of a second subsequence corresponding to positions 116 to 166 of Fuc-TV connected to the amino terminus of a third subsequence corresponding to amino acid positions 161 to 361 of Fuc-TIII;

2. A DNA sequence, comprising a DNA subsequence wherein said DNA subsequence encodes a polypeptide according to Claim 1.

3. A plasmid, comprising a DNA sequence according to Claim 2.



4. A transformed cell, wherein said cell contains a plasmid according to Claim 3.

5. A method of producing a polypeptide, comprising culturing a cell according to Claim 4.



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Figure 2

[illegible]

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Figure 3

10 20 30 40 50 60 70 80 90 100 110  
GAATTCATCGTGGCAAGGGCAGCCTGAATGGATGATGTAACCTGGGCTCTTTCAATGGAGGGCCAGACTCCTGGGTCTAGGGGATGAGGGAGGGGAGGATCGGCTTAGCTGGGAC:

130 140 150 160 170 180 190 200 210 220 230  
GGTGAAGGGGCTGGGGGGCAGATTCTGAGTCTCAGAGAGAAGGATCTGGGCTCTCAAGCACTCAGTGGGAGGGAGGAGGGGCTCTGGGCTCTCGAAAAACCACTCTTGGAC:

250 260 270 280 290 300 310 320 330 340 350  
CTATGCAGATCAGGCAGAACAGAGAAATTTCTGGGGCCATCTGAATTTCTAAGTTTGGGGGAGGGGCTGATCTGACACTGAGGTTCCTTGATCTCAGCAAGGGGCAATTGCT

370 380 390 400 410 420 430 440 450 460 470  
TGAAGAAGGGACCCGATCTGACACACAAGTATCTGGCTTGAAGCTCTCACTGGGCTGGGGCAACCTCAACCTCATCTGCTGCTGAGATGCTCAGACCTGGACATCCC

490 500 510 520 530 540 550 560 570 580 590  
CTCTCTCTCTGATGCAATCTGCTGTTCTTTCAAGAGAGAGCAATCCAGGGCCAGGAGGTGCTCTGAAATAACCTGGGGGAGGGGCTGCTGAAAGTCTCTGACTGGAC:

610 620 630 640 650 660 670 680 690 700 710  
CAGGCAAGGGAGGGCTCGAGTGGGCAAGGAGGGAAGACAGTTGGCTAATTTCTCGAGCCCTAAGGGTCAAGGTAAGGCTTCTGCTGCTGAGGAGGAGGGTGGGCT:

730 740 750 760 770 780 790 800 810 820 830  
ACTCTGCTCTGAGGAGGAGGGCTGGGGGCTGGACTCTGGGTCTGAGGAGGAGGGTCTGGGCTGTACTCTGATCTGAGGAGGAGGGCTGGGAACTGGGCTCT:

850 860 870 880 890 900 910 920 930 940 950  
CTGAGGAGGAGGAGGCTTTGCTCTGACTCTGGGTCTGAGGAGTAGGGCTAGGCACTCTGACTCTGGGTCTGAGGAGGAGGGCTGGGCTCTGACTCTGGGTCTGAG:

970 980 990 1000 1010 1020 1030 1040 1050 1060 1070  
GAGGGGAGGGGCTTGGACTCTGGGTCTGAGGAGGAGGGGGGGGGCTGACTCTTAAGTCTCAGGAGGAGGGTCTGGGGCTGACTCTGGGTCTGAGGAGGAGGGT

1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190  
GTGCTGGGAGTCCGAGGCTGGGAGATGATGCTTAACTTCTGGCAATCAAGTCAAACTCTGAGTCTTGACATTCATGTATCTTCAATGGAGGCTGAGTCTGTGGGAGGA:

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310  
CCAGGTCCGAGGCAAGCACTCAAGGCACAACTGTTTCAGTATAATAAGAAAAATAGTTAGAAATAAGAAATAGTTATCATACAAATTAGATAGAGATGATCATGCAAGATATC

1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430  
ATTAGTGTAACATTATTAATCATTAGCTATTACTTTATTCTTTGTTGTATACTAATATAACGAGGAAACAACCGTGGGTATAGGGTCAGGTACTGAAGGCACATTGTGAGAA

1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550  
CTAGAGGCAAGAGGTGAGCTTCTGTACACGGCATAAGGGCTCTTGAGGCTCTTGGTCAAGCGGCAAGCCAGTGTCTGGAAGGCAACCTTACTCAGGAGACCAGCA

1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670  
CAATCTCTTTTCTTGGAGAGTCAGGGAACACTCTGCTCCAGCTCTTGTGGAGGCTGGGTATTATCTAGGCTGCCCCAGTCATCTCTCTCTCTCTCTCAATGGTC

1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790  
TCTTGTCTCTTGCATTTCTGCGGTACTCTGCTCTCTTTGAGTTCTAGTAGATAGCGGTAGAAGAAATAGTGAAGGCTTTTTTTTTTTTTTTTGGGGGAGTCTC

**Figure 3 (cont'd)**

[illegible]

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Figure 1 (cont'd)

3610 3620 3630 3640 3650 3660 3670 3680 3690 3700 3710 3720  
TCCCCAGCCATTACTCTCAGCAACCAAGATGCTGCTTCCGCTCCTGACCTCCAGCCTCTCTTGTTTACTTGAAGTATCGTTTCCCATCACCACCTCTGCTGCTATTTCCGCTC

3730 3740 3750 3760 3770 3780 3790 3800 3810 3820 3830 3840  
ACACACAGGTACTCCTCAGAACAGGCTGCTGGAAGAGTCCAGTATCAGCCCACTTACAGGAGGGAGACTCGAGATTCTTCAGGAAGGTGTAGCAACCTCCACCACTTTCTTTTT

3850 3860 3870 3880 3890 3900 3910 3920 3930 3940 3950 3960  
TTTTTTTTTTTTTTTAAACAGGCTCCTCTCTGCTCCCAAGCTGAGTGCAGTCAGCGGTGCTATCGCGCTCATTTGTGAGCTCCGGGATCCTCCGCTTAGCATCCGCTGTAG

3970 3980 3990 4000 4010 4020 4030 4040 4050 4060 4070 4080  
CTGAGACACAGCATGTCCACCATGCCAAGCTAATTTATTTATTTTGGAGAGCGGACTTCACTCTTGTTCGCAAGCTCGAGTGTAAATGCCATGATCTCAGCTCAGCGAAC

4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200  
TCCGCGCGCGCGCTTCAGCGCATTTCTCTGCTCAGCTCCCAAGTGGCTGGGATTAAGGCAATGCCGCGCATTTTGTATTTTAAATAGACAGCGTTTCTCCAG

4210 4220 4230 4240 4250 4260 4270 4280 4290 4300 4310 4320  
TTGCTCAGGCTCCTCTCAGCTCCCAAGCTCAGGTGATCCACCACTTGGCTCCCAAGTGGTGGGATACAGGTGTGAGCCAGCGCGCTGGCCATGCCAAGCTAATTTTAAAT

4330 4340 4350 4360 4370 4380 4390 4400 4410 4420 4430 4440  
TTTTTGAAGAGTCTCTTTGCCAGGCTGATCTTCAACTCTGCGCTCAAGGATCTCCCATCTCAGCTCCCAATATGCTGGATTAAGGTGTGAGCCAGCTGCCACCAAG

4450 4460 4470 4480 4490 4500 4510 4520 4530 4540 4550 4560  
CATGGCTATCTCAAAACCACTTCTTCTCAGCTCCCATGCCGAAATTCAGGCTCTCATCCTCAAGCTAGGACTCAGGCTCTCCTACCTCAGCCCAAGGAGTGTAAAGCTTTA

4570 4580 4590 4600 4610 4620 4630 4640 4650 4660 4670 4680  
ACTTCTCTTTTCCCTGGCACTAAGGAGTCTGCACCCAGGCGCTCCTTACCCGACATCCTCTCAGCTCCGCTCTCAGGCTCAGTGAATTTGCTAATTCGCTTTCTGCGCTC

4690 4700 4710 4720 4730 4740 4750 4760 4770 4780 4790 4800  
CAGCAATGTGCTCCGACCACTCCTCAGCTCTGCTGCGCTTCTCTAGTCTGTGCTCTCTGTAAATCTTCTCTCCATATCCATCAAGACAGCTTTCACATGGCTAGCGCTGT  
MetIrpLeuArgSerHisArgGlnLeuCysLeuAlaPheLeuLeuValCysValLeuSerValIlePhePheLeuHisIleHisGlnAspSerPheProHisGlyLeuGlyLeu

4810 4820 4830 4840 4850 4860 4870 4880 4890 4900 4910 4920  
CGATCTGTGCTCAGACCGCGCTGCTGACACCCCAAGTGGCACTTTCTGCTGCGCGGACTGGATGGCGCCCAAGCTCTCTTCTCTGCTCCAGACCTGCTTCTCTCTCC  
erIleLeuCysProAspArgArgLeuValThrProProValAlaIlePheCysLeuProGlyThrAlaMetGlyProAsnAlaSerSerSerCysProGlnHisProAlaSerLeuSerG

4930 4940 4950 4960 4970 4980 4990 5000 5010 5020 5030 5040  
GCACCTGGACTGTCTACCCCAATGCCGCTTTGTAATCAGATGGCAGATATGCCAGCTGCTGCTCTGCGCCAGCTCAAGCGCGCGCGGCTTTATCTGCTGCTGCCATGCATGCC  
lyIhrIroIhrValIyrProAsnGlyArgPheGlyAsnGlnMetGlyGlnIyrAlaIhrLeuLeuAlaLeuAlaGlnLeuAsnGlyArgArgAlaPheIleLeuProAlaMetHisAla

5050 5060 5070 5080 5090 5100 5110 5120 5130 5140 5150 5160  
CCTGCGCGCGCTATTCGCACTACCTGCGCTGCTGCGCCAGAGTGGACAGCGCGCGCGCTGCGCGAGCTCCAGCTTCAAGCTGGATGCGGAGGAGTACCGGACTTCAGAG  
IleLeuAlaProValPheArgIleThrLeuProValLeuAlaProGlyValAspSerArgIhrProIhrArgGlyLeuGlnLeuHisAspIhrMetSerGlyGlyIyrAlaAspLeuArg

5170 5180 5190 5200 5210 5220 5230 5240 5250 5260 5270 5280  
ATCTTTCTGAGCTCTCTGCTTCCGCTCTTGGACTTTCTTCACCATCTCCGCAAGAGATCCGAGAGGTTACCTTCAGCAGCAGCTTCCGGAAGAGCGGAGAGTGTGC  
SDProPheLeuLeuValLeuSerGlyPheProCysSerIhrIhrPhePheHisIleLeuArgGlyGlnIleArgArgGlyPheThrLeuHisAsnHisLeuArgGlyGlyAlaGlnSerVal

5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400  
TGGTCAGCTCCGCTGCGCGCCAGCGGACCGCGCGCGGACTTTGCTGCGCTCCAGCTGCGCGGCGGACTATCTGAGGTTATGCTCAGCGCTGCAAGGCTGCTGCGCGGAC  
eGlyGlnLeuArgLeuGlyArgIhrGlyAspArgProArgIhrPheValGlyValHisValArgArgGlyAspIyrLeuGlnValMetProGlnArgIhrPheGlyValValGlyAsp



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Figure 3 (cont'd)

7210 7220 7230 7240 7250 7260 7270 7280 7290 7300 7310 7320  
GGTCTTCTCTCTTTCACAGTCTCTGAAAAAGTGGGCTAGTTGGTGAGCAGACCCGCTCTGTCACAAAGCCCCACAGCCCTCAAAGACTTTTTTTTGTGTTTTGAGCAGACAG

7330 7340 7350 7360 7370 7380 7390 7400 7410 7420 7430 7440  
GCTAAATGTGAACGTGGGTGAGGATCACTGCCAAATCGTACAGCTTCTGCAGCAGAACTTCCAGGATCCAGGCACACTTTTTTAAAGCTATAAACTGCCAAGAGCTGATA

7450 7460 7470 7480 7490 7500 7510 7520 7530 7540 7550 7560  
TATTGGGTGTGAGTTCAGGTTGGCTCTCACAATGAAGGAAGTGGCTCTTGTCTGCAGGTGGGCTGCTGAGGCTCTGGGATCTGTTTTCTGGAAGTGTGCAGGTATAACACACCCCTCTG

7570 7580 7590 7600 7610 7620 7630 7640 7650 7660 7670 7680  
TGGTGTGCAAACTGCCAGGTACCGTGTCTCAATTGCTAACCAGTCTGTCTGCTCAACTCCAGAACCACTACATCTGGCTTTGCCAGGTCTGAGATAAAACATCTAAAGGTAGCCAG

7690 7700 7710 7720 7730 7740 7750 7760 7770 7780 7790 7800  
ACCTGGAGCCAGCTCAGATCCAGGCAGGAGCAGGAGGTCTGCCAAGGTCCAGCCGTTGTCAGATCTCAGAGCCGCTTCTGTTTTTTCAGCGGTAAAGAGAAAGCTTAAACA

7810 7820 7830 7840 7850 7860 7870 7880 7890 7900 7910 7920  
TASTCAGCTCTGATCAGATCCCTGTCTACTCATCCAGACCCGATGCTGTAGCTTATCAGGAGTTACAGTTACAAATTGTTACAGTACTGTTCCCACTCAGTCCACCGGTGAGAG

7930 7940 7950 7960 7970 7980 7990 8000 8010 8020 8030 8040  
AGCAGCAGGTATGAATTAAGTCTACAGCACTAACCGTGTCTCTGTAGCTTTTTTCCAGCCAGCCACTGTGTATGTGTGTGGGTTTGTGTGTGTGTGTGTGTGTGTGTGT

8050 8060 8070 8080 8090 8100 8110 8120 8130 8140 8150 8160  
AAGAGGTCCAGAAAAAGGTGGGCTACTTCTGAAGACTTTTATTTTTTTTAAATTAATTTATTTTTTTCAGAGATCCAGTCTTCTCTGTGCCCCAGGCTGAGTGCAGTACTGTGATC

8170  
TCCGCTCACTGCAA



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Figure 4

10	20	30	40	50	60
CTGCA	GAGAG	CCCCA	CCCCG	AAGCC	ACTTT
TATAG	AAGCT	TTTAC	ACACA	ATGCT	TCATT
70	80	90	100	110	120
TTTTT	TTTTT	TTTTT	CGAGA	CGGAG	TCTCG
CTTTC	TGCCC	CAGGC	TGGAG	TGCAG	TGCCC
130	140	150	160	170	180
CGATC	TGCCC	TCACT	GCAAG	CTCCG	CTCTC
TGGGT	TGACG	GCATT	CTCCT	GCCTC	AGCTT
190	200	210	220	230	240
CCCCA	GTAGC	TGGGA	CTACA	GGCCC	GGCCC
ACCAA	GGCTG	GCTAA	TTTTT	TTTTA	TTTTT
250	260	270	280	290	300
AGTGG	AGACA	GAGTT	TGACC	GTCTT	AGCCA
GGATG	GTCTC	GATCT	CTCTA	CTCTG	GGATC
310	320	330	340	350	360
CGCCC	GGCTC	GGCTT	CCCAA	AGTGC	TGGGA
GTATA	GGGCT	GAGCC	ACCCC	GGCTG	GGCTA
370	380	390	400	410	420
TACTT	GATTT	TTAAT	GAAAA	CATTC	TTAAA
TTCAT	ATGGC	TAACG	CAAAAT	TTATT	TTCTG
430	440	450	460	470	480
TAGGC	ATAAC	ATCAA	AAACA	CTCTG	CAGGA
CTCCC	GCATT	CCCAG	CATCT	TCTAG	TTCTC
490	500	510	520	530	540
CCCTA	GTATC	AGTCC	CATCT	CATCT	ATCCA
CAGCT	GTGAT	CTACT	AAAAC	TTTTT	TCAAA
550	560	570	580	590	600
ACTTT	GTCTT	GTCTT	TAGGT	CAGCA	GGCCC
GGCCC	TCATC	TATTT	GGAAA	TGCCC	TGAAT
610	620	630	640	650	660
AAAAG	TTGAA	TATCA	TAAAC	CAAAG	CGAAC
ACCCA	GAAAT	TCAAA	TTCAA	CCGCT	AGGTA
670	680	690	700	710	720
AAAAA	TTTTT	CAAGT	CATCT	TAGAC	GTAGA
TCTCT	CCAGT	GTCCC	CTAAT	AAGGT	AGAAG
730	740	750	760	770	780
AGCCC	AGTCC	CATAC	TCTCT	TTACA	CCCTT
AACTT	GGCTG	CTAGA	ATATT	TATCT	TGCTC
790	800	810	820	830	840
ATCAT	TTTAT	CATCC	AAACT	ATTTT	GCATA
ACTTT	CATGG	GTCGA	GAAAA	TTTTT	TTTAA
850	860	870	880	890	900
GTGCT	TGCTA	AAATT	AAIAG	TCATA	TTCAT
TCATT	CATCT	CATCT	AACAG	GCAAT	AAATT

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Figure 4 (cont'd)

910	920	930	940	950	960
CGTTC	ACGAC	AAGGG	CGTTC	GGGGG	GGCCA
CATCT	TCATC	TTTGG	TTTAT	GAGTC	CTGTC
970	980	990	1000	1010	1020
CGTCT	TGCTA	CAAGC	AATAC	TACTA	TGAGC
CGCCA	AGTCA	GACTT	ATTTC	GTAGG	GGACC
1030	1040	1050	1060	1070	1080
AAAGG	AAAGA	ACATG	TTTTG	ATTTC	TARGA
AAACA	TTTTG	TTCTC	TATCC	TTTAC	TGGCC
1090	1100	1110	1120	1130	1140
TGGCA	GGCAA	AGGAA	ATGTT	CTTAT	GAGCA
CTCAC	ATTGA	AAACT	TAACT	TCTTC	ACCAA
1150	1160	1170	1180	1190	1200
ATCCA	GAGAC	TTTCA	AGGCG	ATGCG	CGTCC
CGGCT	CGCTC	CACAA	TTTCA	CGCTC	TGGCC
1210	1220	1230	1240	1250	1260
GGGCC	AGGAG	ATGCT	GGCCA	CGGAT	CGGCT
GGGCG	CGGCT	CGCTC	CACAA	TTTCA	CGCTC
1270	1280	1290	1300	1310	1320
CGTCT	GGACT	CGCTC	CGTCC	CTCAA	TGCTT
CGCTC	CGGCG	CGGCT	CGCTC	CGGCG	CGGCT
1330	1340	1350	1360	1370	1380
AGGCC	CAACG	CGAGG	AAGCC	GAGCC	TATCC
TGCTT	TGCTT	TGCTT	GGGCG	CGGCT	CGGCG
1390	1400	1410	1420	1430	1440
TCTGT	TCTAG	GGGCT	CGTCC	TGCGC	GGCAG
CTGCT	TTAGA	AGGTC	TGAGG	CGTCC	TGTAC
1450	1460	1470	1480	1490	1500
CTTCC	GAGGG	ATGAA	CGGCG	CGTCC	CGGCT
CGAAG	CGGAG	CGTCC	CGGCG	ACAGT	GAGCC
1510	1520	1530	1540	1550	1560
AGGCC	GAGGG	CGGTC	GGGCG	GGCCA	GAGGG
AAACC	GGATC	AGTTG	AGAGA	GAATC	AAGAG
1570	1580	1590	1600	1610	1620
TAGCC	GATGA	GGGCG	TTGTC	GGGCG	GGGCG
GGGCG	GGGCG	GGGCG	GGGCG	GGGCG	GGGCG
1630	1640	1650	1660	1670	1680
GAGTC	GGGCG	AGGCC	GGCCA	GGAGG	CTGCT
GGGCG	CGGCT	CGGCG	CGGCT	GGGCG	CGGCG
1690	1700	1710	1720	1730	1740
CGCAG	TGGAA	GAAAG	GGAGG	GGGCG	TGCTT
GGGCT	CGTCC	CGGCG	CGGCG	CGGCG	CGGCG
1750	1760	1770	1780	1790	1800
CTGCG	CGGTC	GGGCG	GGGCG	GGGCG	GGGCG
GGGCG	GGGCG	GGGCG	GGGCG	GGGCG	GGGCG

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Figure 4 (cont'd)

1810	1820	1830	1840	1850	1860
TCTGG	GACGG	GGGGG	TTCCA	TTTCC	GGGGG
AGCGGG	CGAGC	GGCAG	CGAGC	GGCTGG	AGCGGG
1870	1880	1890	1900	1910	1920
CAGCT	ACAGC	ATGAG	AGCGG	GTGGC	GGCTTC
TCCAC	GGCTG	CGGAC	GGCTG	GGCAG	CGGAG
1930	1940	1950	1960	1970	
GCAGC	GGTTC	GTGTT	CGGCG	ATG	GGG
GCA	CCG	TGG	GGC	TGG	CCG
ACC	GGC	GGC	ACC	GGC	GGC
Mor Gly Ala Pro Trp Gly Ser Pro Thr Ala Ala					
1980	1990	2000	2010	2020	
GGC	GGC	GGC	GGC	GGC	GGC
TGG	CCG	CGA	GGC	GGC	GGC
GTG	CCA	TGG	ACC	GGC	GGC
Ala Gly Gly Arg Arg Gly Trp Arg Gly Arg Gly Leu Pro Trp Thr Val Cys					
2030	2040	2050	2060	2070	2080
GTG	GTG	GGC	GGC	GGC	GGC
TTG	ACC	TGT	ACC	GGC	GTG
ATC	ACC	TAC	GGT	TGG	TGG
Val Leu Ala Ala Ala Gly Leu Thr Cys Thr Ala Leu Ile Thr Tyr Ala Cys Trp					
2090	2100	2110	2120	2130	
GGG	GAG	GTG	GGC	GGC	GGC
TGG	GGC	TGG	CCA	ACC	GGC
TGG	CCA	CCG	GGC	GGC	GGC
Gly Gln Leu Pro Pro Leu Pro Trp Ala Ser Pro Thr Pro Ser Arg Pro Val Gly					
2140	2150	2160	2170	2180	2190
GTG	GTG	GTG	TGG	TGG	GAG
CCC	TTT	GGG	GGG	GGC	GAT
AGC	GGC	CCG	AGG	CCG	AGG
Val Leu Leu Trp Trp Glu Pro Phe Gly Gly Arg Asp Ser Ala Pro Arg Pro Pro					
2200	2210	2220	2230	2240	
CCT	GAC	TGG	GGC	GTG	GGC
TTT	AAC	ATC	AGC	GGC	TGG
GGC	GGC	GGC	GGC	GGC	GGC
Pro Asp Cys Pro Leu Arg Phe Asn Ile Ser Gly Cys Arg Leu Leu Thr Asp Arg					
2250	2260	2270	2280	2290	
GGG	TGG	TAC	GGA	GAG	GGT
CAG	GGC	GTG	CTT	TTT	GAC
CAC	GGC	GAC	GGC	GAC	GGC
Ala Ser Tyr Gly Glu Ala Gln Ala Val Leu Phe His His Arg Asp Leu Val Lys					
2300	2310	2320	2330	2340	2350
GGG	GGC	GGC	GAC	TGG	GGC
GGC	GGC	GGC	GGC	GGC	GGC
ATC	CAG	GGC	CAC	ACT	GGC
Gly Pro Pro Asp Trp Pro Pro Pro Trp Gly Ile Gln Ala His Thr Ala Glu Glu					
2360	2370	2380	2390	2400	
GTG	GAT	GTG	GGC	GTG	TTT
GAC	TAC	GAG	GAG	GCA	GGC
GGC	GGC	GGC	GGC	GCA	GAA
Val Asp Leu Arg Val Leu Asp Tyr Glu Glu Ala Ala Ala Ala Glu Ala Leu					
2410	2420	2430	2440	2450	2460
GGG	ACC	TGG	AGC	GGC	AGG
GGC	GGC	GGC	GGC	GGC	GGC
GAG	GGC	TGG	GGT	TGG	ATG
Ala Thr Ser Ser Pro Arg Pro Pro Gly Gln Arg Trp Val Trp Met Asn Phe Glu					
2470	2480	2490	2500	2510	
TGG	GGC	TGG	GAC	TGG	GGC
GGG	GTG	CCA	AGC	GTG	GCA
AGT	AAC	GTG	TTT	AAC	TGG
Ser Pro Ser His Ser Pro Gly Leu Arg Ser Leu Ala Ser Asn Leu Phe Asn Trp					

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Figure 4 (cont'd)

2520	2530	2540	2550	2560	
ACG CTC TCC TAC CCG GCG GAC TCC GAC GTC TTT GTG CTT TAT GCG TAC CTC TAC					
Thr Leu Ser Tyr Arg Ala Asp Ser Asp Val Phe Val Pro Tyr Gly Tyr Leu Tyr					
2570	2580	2590	2600	2610	2620
CCC ACA AGC GAC CCG GCG GAC CCG CCC TCA GCG CTC GCG CCG CCA CTC TCC AGG					
Pro Arg Ser His Pro Gly Asp Pro Pro Ser Gly Leu Ala Pro Pro Leu Ser Arg					
2630	2640	2650	2660	2670	
AAA CAG CCG CTC GTG GCA TCG GTG GTG AGC CAC TCG GAC GAG CCG CAG GCG CCG					
Lys Gln Gly Leu Val Ala Trp Val Val Ser His Trp Asp Glu Arg Gln Ala Arg					
2680	2690	2700	2710	2720	2730
GTG CCG TAC TAC GAC GAA CTC AGC GAA CAT GTG ACC GTG GAC GTG TTC GCG CCG					
Val Arg Tyr Tyr His Gln Leu Ser Gln His Val Thr Val Asp Val Phe Gly Arg					
2740	2750	2760	2770	2780	
GGC CCG CCG CCG GAG CCG GTG CCG GAA ATT GCG CTC CTC CAC ACA GTG GCG CCG					
Gly Gly Pro Gly Gln Pro Val Pro Gln Ile Gly Leu Leu His Thr Val Ala Arg					
2790	2800	2810	2820	2830	
TAC AAG TTC TAC CTC GCT TTC GAG AAC TCG CAG CAC CTC GAT TAT ATC ACC GAG					
Tyr Lys Phe Tyr Leu Ala Phe Gln Asn Ser Gln His Leu Asp Tyr Ile Thr Glu					
2840	2850	2860	2870	2880	2890
AAG CTC TCG CCG AAC CCG TTC CTC GCT GCG CCG GTG CCG GTG GTG CTC GCG CCA					
Lys Leu Trp Arg Asn Ala Leu Leu Ala Gly Ala Val Pro Val Val Leu Gly Pro					
2900	2910	2920	2930	2940	
GAC CCG CCG AAC TAC GAG CCG TTT CTC CCG CCG GCG CCG TTC ATC CAC CTC GAC					
Asp Arg Ala Asn Tyr Glu Arg Phe Val Pro Arg Gly Ala Phe Ile His Val Asp					
2950	2960	2970	2980	2990	3000
GAC TTC CCA AGT GCG TCC TCC CTC GCG TCG TAC CTC CTT TTC CTC GAC CCG AAC					
Asp Phe Pro Ser Ala Ser Ser Leu Ala Ser Tyr Leu Leu Phe Leu Asp Arg Asn					
3010	3020	3030	3040	3050	
CCC GCG CTC TAT CCG CCG TAC TTC CAC TCG CCG CCG AGC TAC GCT CTC CAC ATC					
Pro Ala Val Tyr Arg Arg Tyr Phe His Trp Arg Arg Ser Tyr Ala Val His Ile					
3060	3070	3080	3090	3100	
ACC TCC TTC TCG GAC GAG CCG TCG TCG CCG CTC TCG CAG GCT GTA CAG AGG GCT					
Thr Ser Phe Trp Asp Glu Pro Trp Cys Arg Val Cys Gln Ala Val Gln Arg Ala					
3110	3120	3130	3140	3150	3160
GGG GAC CCG CCG AAG AGC ATA CCG AAC TTC GCG AGC TCG TTC GAG CCG TGA A					
Gly Asp Arg Pro Lys Ser Ile Arg Asn Leu Ala Ser Trp Phe Gln Arg ***					

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Figure 4 (cont'd)

3170	3180	3190	3200	3210	3220
GGGGC	GCTCC	GCTGG	AAGCC	ACCCA	GCGGA
GCGCA	AGTTG	TCAGC	TTTTT	GATCC	TCTAC
3230	3240	3250	3260	3270	3280
TGTGC	ATCTC	CTTCA	CTGCC	GCATC	ATGCG
AGTAA	GTTCT	TCAAA	CACCC	ATTTT	TCTTC
3290	3300	3310	3320	3330	3340
TATCG	GAAAA	AAACG	ATTTA	CCAAT	TAATA
TTACT	CAGCA	CAGAG	ATGGG	GGCCC	GCTTT
3350	3360	3370	3380	3390	3400
CCATA	TTTTT	TGCAC	AGCTA	GCAAT	TGGCC
TGCGT	TTCCT	GCTCA	TGGCC	ATCAT	TCTTT
3410	3420	3430	3440	3450	3460
AGGGC	TGAAC	GAGGG	GCTTC	TTTTT	CAGCT
TGTAA	CCAGT	GCAGA	AATGA	AATAG	CTTAG
3470	3480	3490	3500	3510	3520
CGGCA	AGAAG	CGCTT	GAGGC	GCTTT	GCTGA
ATTTG	CCCAT	CTGCC	ACAGG	CCATA	TTTTT
3530	3540	3550	3560	3570	3580
GGCCC	GTCCA	GCTTC	CAAAAT	CTCAT	ACACA
ACTGT	TCCCG	ATTCA	CGTTT	TTCTG	GACCA
3590	3600	3610	3620	3630	3640
AGGTG	AAGCA	AATTT	GTGCT	TGTAG	AAGGA
GCCTT	GTTGG	TGGAG	AGTGG	AAGGA	CTGTC

GCTCC AG



**FIGURE 6**

[illegible]

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Figure 7 (page 1)

α(1,3)FT DNA (-128)	TTTTCTCA
α(1,3)FT DNA (-120)	TCTGTGAACAGGAATATAACAGCTCTTCTCAGGACTCATGGCCTGGAGCTTTGGTAAG
α(1,3)FT DNA (-60)	CAGGAGATTGTCATCARTGACCCCTCACTCTCTCCCCACTTCCCAGAGACTCTGACCC
α(1,3)FT AA	M D P L G P A K P Q W S W R C C L T T L
α(1,3)FT DNA (11)	ATGGATCCCTGGGCCCGGCCAAGCCACAGTGGTGGCGCTGCTGCTGACCCACGCTG
Lewis FT DNA	       ATGGATCCCTGGGTGCAGCCCAAGCCACAAATGGCCATGGCGCGCTGCTGGCGGCACCTG
α(1,3)FT AA	L F Q L L M A V C F F S Y L R V S Q D D
α(1,3)FT DNA (61)	CTGTTTCAGCTGCTGATGGCIGTGTTCTTCTCTCTATCTGCGTGTGCTCAAGACGAT
Lewis FT DNA	       CTATTCAGCTGCTGGTGGCTGTGTGTTCTTCTCTCTACCTGCGTGTGCTCCCGGAGACGAT
α(1,3)FT AA	P T V Y P N G S R F P D S T G T P A H S
α(1,3)FT DNA (121)	CCCAGTGTGTACCCCTAATGGTCC...CGCTTCCCA..GACAGCACAGGGACCCCGCCACTCC
Lewis FT DNA	       GCCACTGGATCCCTAG.GGCTCCCAAGTGGGTCTCTCCGACAGGACACC.ACTCCACCCGCCCC
α(1,3)FT AA	I P L I L L W T W P F N K P I A L P R C
α(1,3)FT DNA (181)	ATCCCCCTGATCCTGCTGTGGACGTGGCCTTTTAAACAACCCCATAGCTCTGCCCGCGCTGC
Lewis FT DNA	       ACCCCTCCTGATCCTGCTATGGACATGGCCTTTCACATCCCTGTGGCTCTGTCCCGCTGT
α(1,3)FT AA	S E M V P G T A D C N I T A D R K V Y P
α(1,3)FT DNA (241)	TCAGAGATGGTGGCTGGCAGCGGTGACTGCARACATCACTGCCGACCGCAAGGTGTATCCA
Lewis FT DNA	       TCAGAGATGGTGGCCCGGCACAGCCGACTGCCACATCACTGCCGACCGCAAGGTGTATCCA



(U), JIFT AA      Q A D A V I V II R E V M Y N P S A Q L  
 (U), JIFT DIA (DII) CAGGCAGCGGGTCATCTGACCCACCAGAGGTTCATGTACAAACCCAGTGCCCAAGTC  
 ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||  
 (Lys T DIA) CAGGCAGACACGGTTCATCTGTGCACCACCTGGGATNTCATGTCCAACCCCTAAGTCACGCCCTC

[illegible][illegible]

u(1), JIFT AA	D S D I F T P Y G W L E P W S G Q P A H
u(1), JIFT DNA (481)	GACTCGGACATCTTCACGGCCCTACGGCTGGCTGGAGCCGTGGTCCGGCCAGCCTGCCCCAC       GACTCGGACATCTTCACGGCCCTACGGCTGGCTGGAGCCGTGGTCCGGCCAGCCTGCCCCAC
Lys-9, IT DNA	

u11, 31FT AA	P P L N L S A K T E L V A W A V S N W G
u11, 31FT DNA (561)	CCACCGCTCAACCTCTCGGCCAAGACCAGGCTGGTGGCTGGGCAGTGTCCAACTGGGGG       CCACCGCTCAACCTCTCGGCCAAGACCAGGCTGGTGGCTGGGCAGTGTCCAACTGGGAAG
u11s 31T DNA	

[illegible]

Figure 7 (page 3)

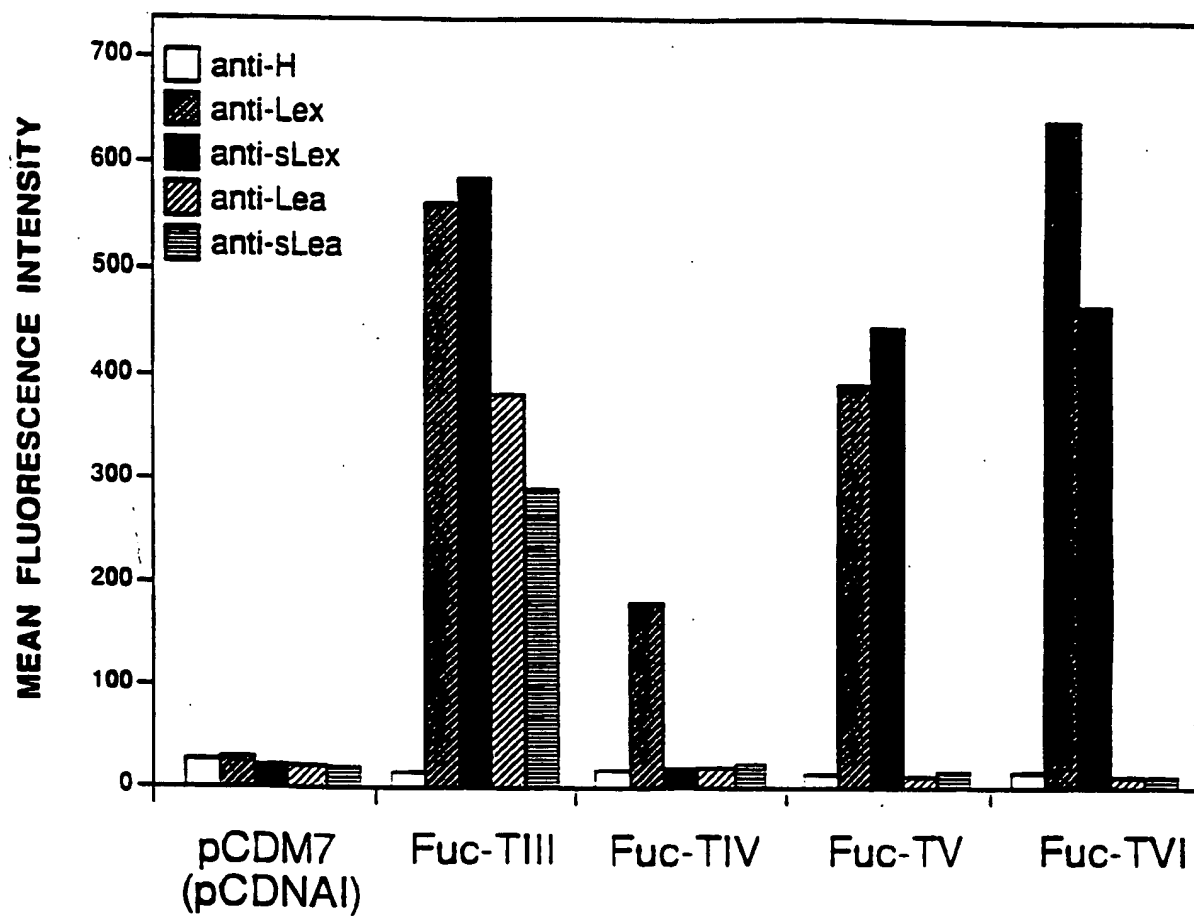
α(1,3)FT AA	Y G R S H K P L P Q G T M M E T L S R Y
α(1,3)FT DNA (661)	TACGGACGCTCCACAAAGCCCTGCCCCAGGGAACCATGATGGAGACGCTGTCCCGGTAC
LowIs FT DNA	
	TACGG/CGCTCCACAAAGCCCTGCCCAAGGGGACCATGATGGAGACGCTGTCCCGGTAC
α(1,3)FT AA	K F Y L A F E N S L H P D Y I T E K L W
α(1,3)FT DNA (721)	AAGTTCTATCTGGCCCTTCGAGAACTCCTTGCACCCCGACTACATCACCGAGAGCTGTGG
LowIs FT DNA	
	AAGTTCTACCTGGCCCTTCGAGAACTCCTTGCACCCCGACTACATCACCGAGAGCTGTGG
α(1,3)FT AA	R H A L E A W A V P V L G P S R S H Y
α(1,3)FT DNA (781)	AGGAACGCCCTGGAGGCCCTGGGCCGTGCCCGTGGTGGTGGGCUCCAGCAGAAGCAACTAC
LowIs FT DNA	
	AGGAACGCCCTGGAGGCCCTGGGCCGTGCCCGTGGTGGTGGGCUCCAGCAGAAGCAACTAC
α(1,3)FT AA	E R F L P P D A F I H V D D F Q S P K D
α(1,3)FT DNA (841)	GAGAGGTTCCCTGCCACCCGACGCCCTTCATCCACGTGGACGACTTCCAGAGCCCCAAGGAC
LowIs FT DNA	
	GAGAGGTTCCCTGCCACCCGACGCCCTTCATCCACGTGGACGACTTCCAGAGCCCCAAGGAC
α(1,3)FT AA	L A R Y L Q E L D K D H A R Y L S Y F R
α(1,3)FT DNA (901)	CTGGCCCGGTACCTGCAGGAGCTGGACAAAGGACCACGCCCGCTACCTGAGCTACTTTTCGC
LowIs FT DNA	
	CTGGCCCGGTACCTGCAGGAGCTGGACAAAGGACCACGCCCGCTACCTGAGCTACTTTTCGC
α(1,3)FT AA	W R E T L R P R S F S W A L A F C K A C
α(1,3)FT DNA (961)	TGGCGGGAGACGCTGGGCCCTCGCTCCTTCAGCTGGGCACTCGCTTTCGCAAGGCCCTGC
LowIs FT DNA	
	TGGCGGGAGACGCTGGGCCCTCGCTCCTTCAGCTGGGCACTCGCTTTCGCAAGGCCCTGC
α(1,3)FT AA	W K L Q E E S R Y Q T R G I A A W F T Stop
α(1,3)FT DNA (1021)	TGGAAACTGCAGGAGGAATCCAGGTACCAAGACACCGCGC...ATAGCGGCTTGGTTACACCTGA
LowIs FT DNA	
	TGGAAACTGCAGCAGCAATCCAGGTACCAAGACGCGTGGCGCAGCATAGCGGCTTGGTTACACCTGA

Figure 7 (page 4)

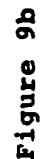
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GGGGCCCTCATCTACCTAAGGACTCGTTGGCCTGAAGCTTCACCTGCCCTGAGGACTCACCT  
GCCTGGGACGGTCACTGTTCAGCTTCACCTGCCCTGGGATTACCTACCTGGGTCCCTC  
ACTTTCCTGGGGCCCTCACCTGCTGGAGTCTTCGGTGGCCAGGTATGTCCCTTACCTGGGA  
TTTTCACATGCTGGCTTCCAGGAGCGTCCCTGGGAGCCCTGGCCTGCTGGGATGTCTC  
CTGGGACTTTGCCTACTGGGGACCTCGGCTGTTGGGACTTTACCTGCTGGGACCTGCT  
CCGAGAGACCTTCCACACTGANTCTCACCTGCTAGGAGCCCTCACCTGCTGGGGACCTCAC  
CCTGGAGGCACCTGGGGCCCTGGGAACT

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Figure 8.



**Figure 9:**



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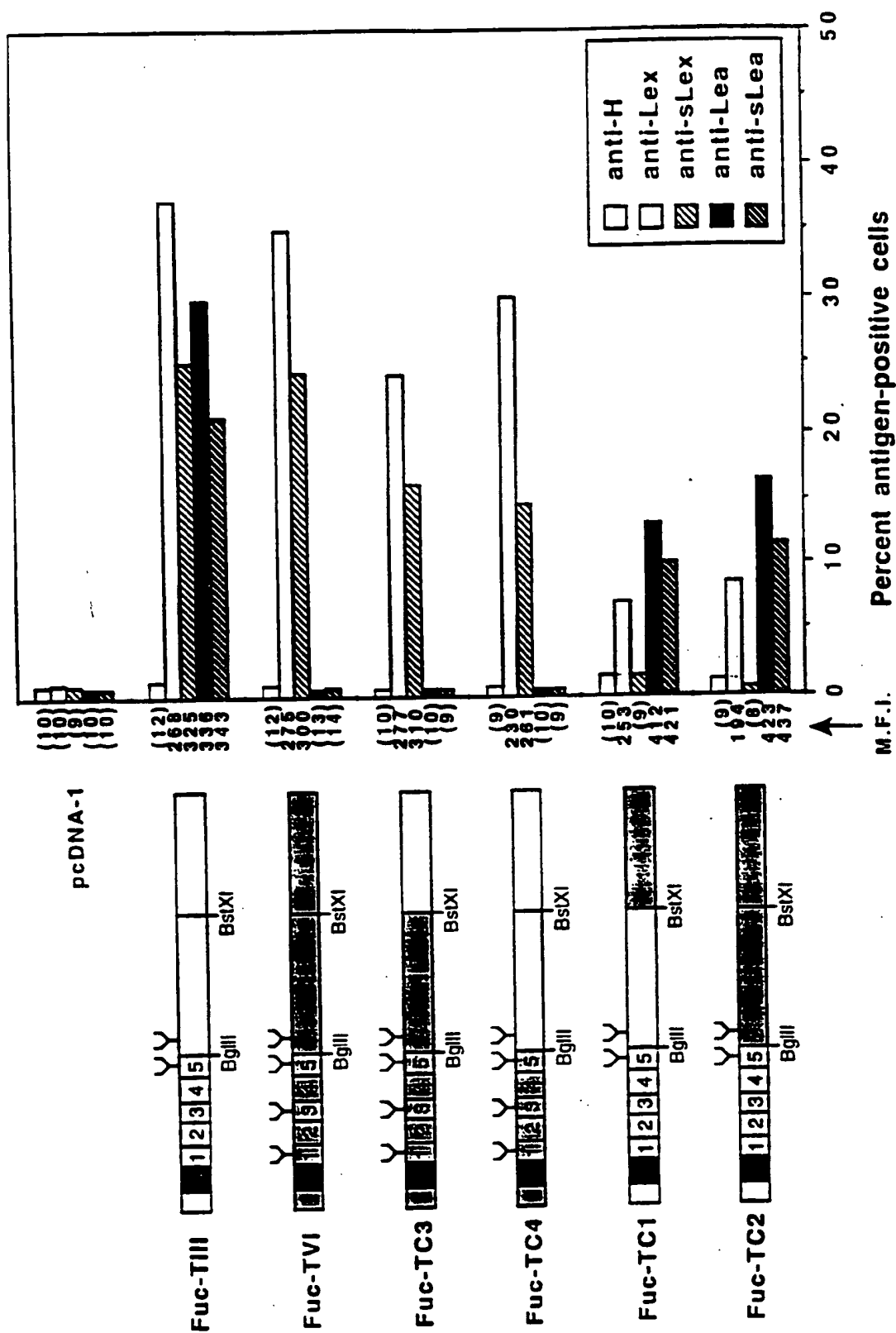


Figure 10

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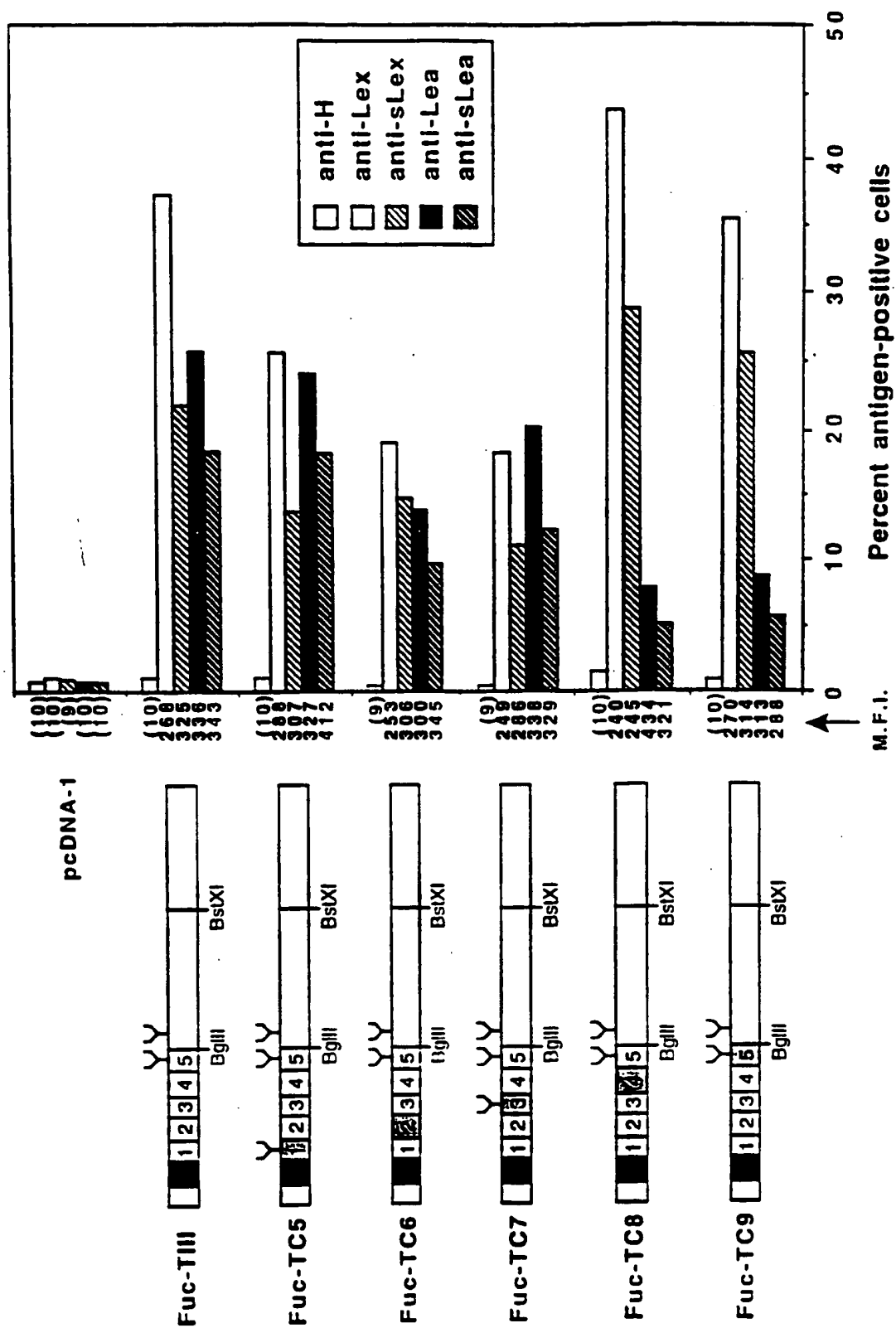


Figure 11a



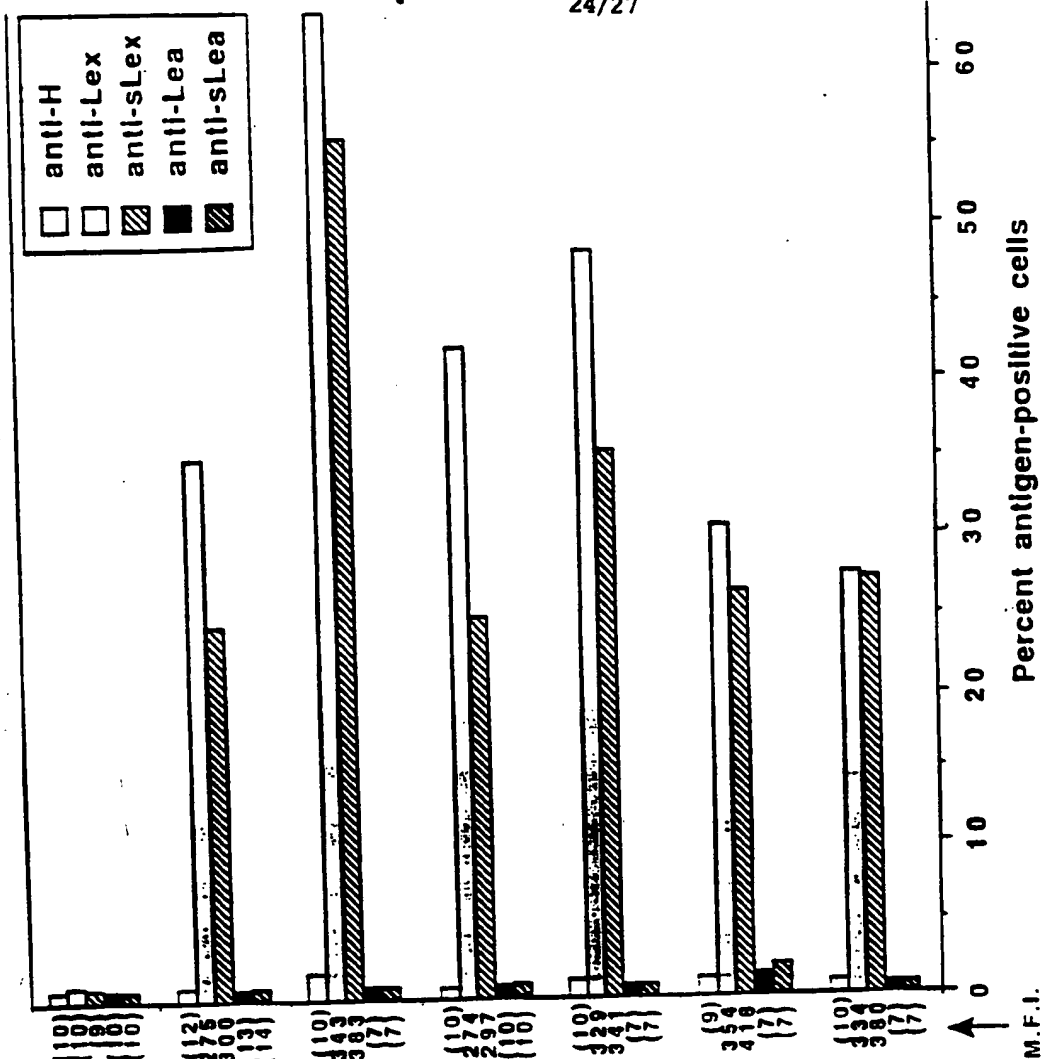
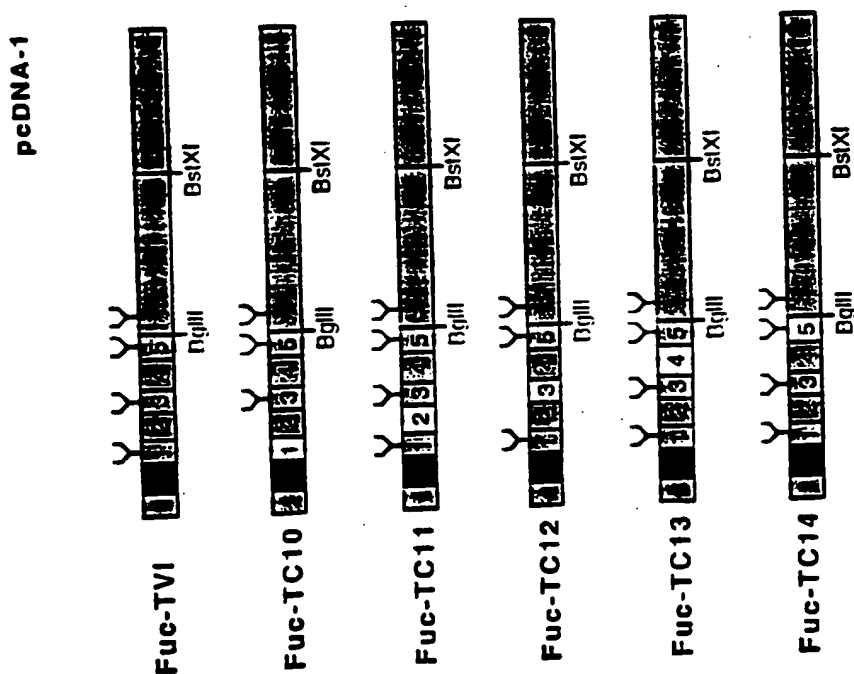
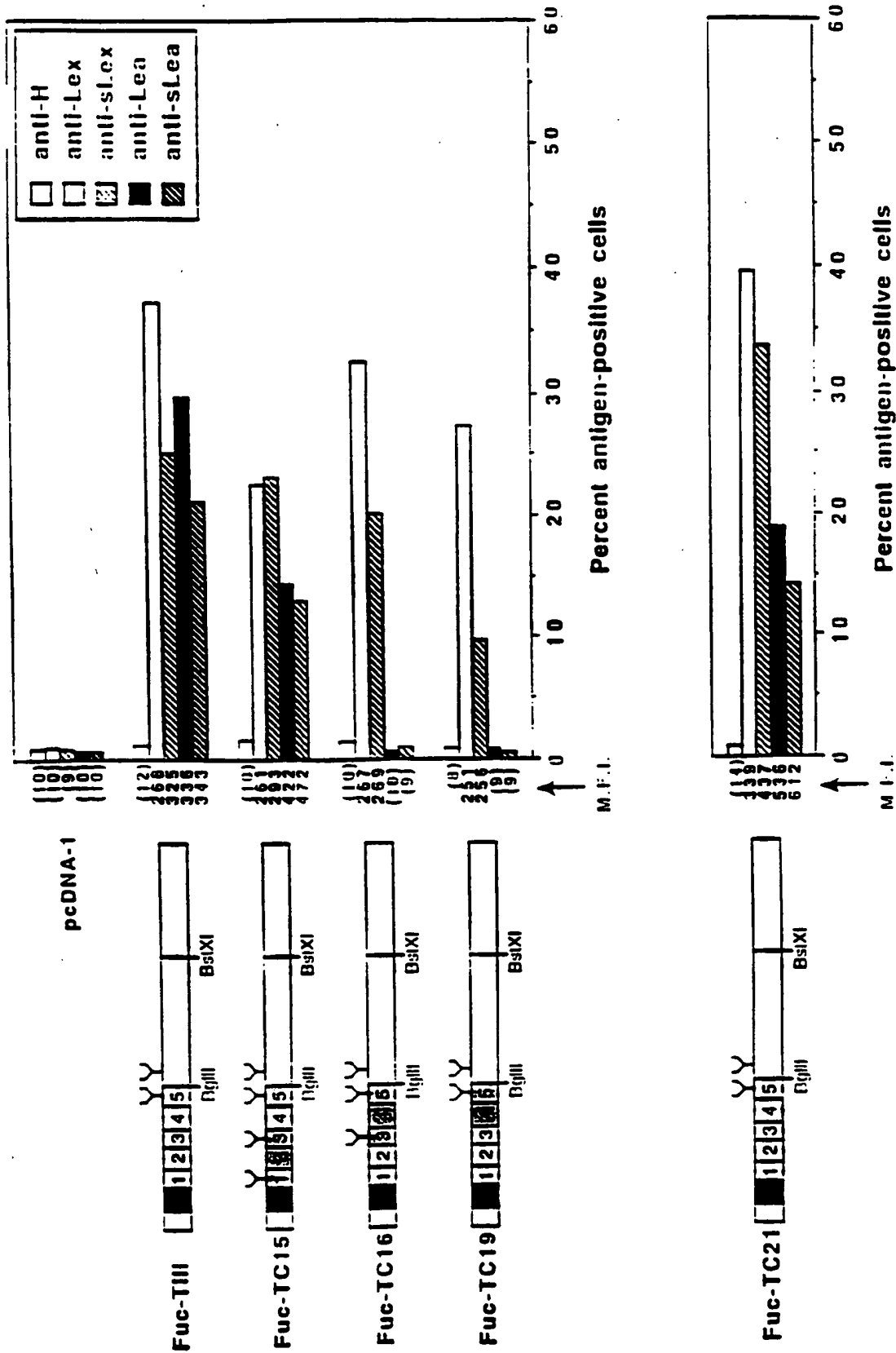


Figure 11b



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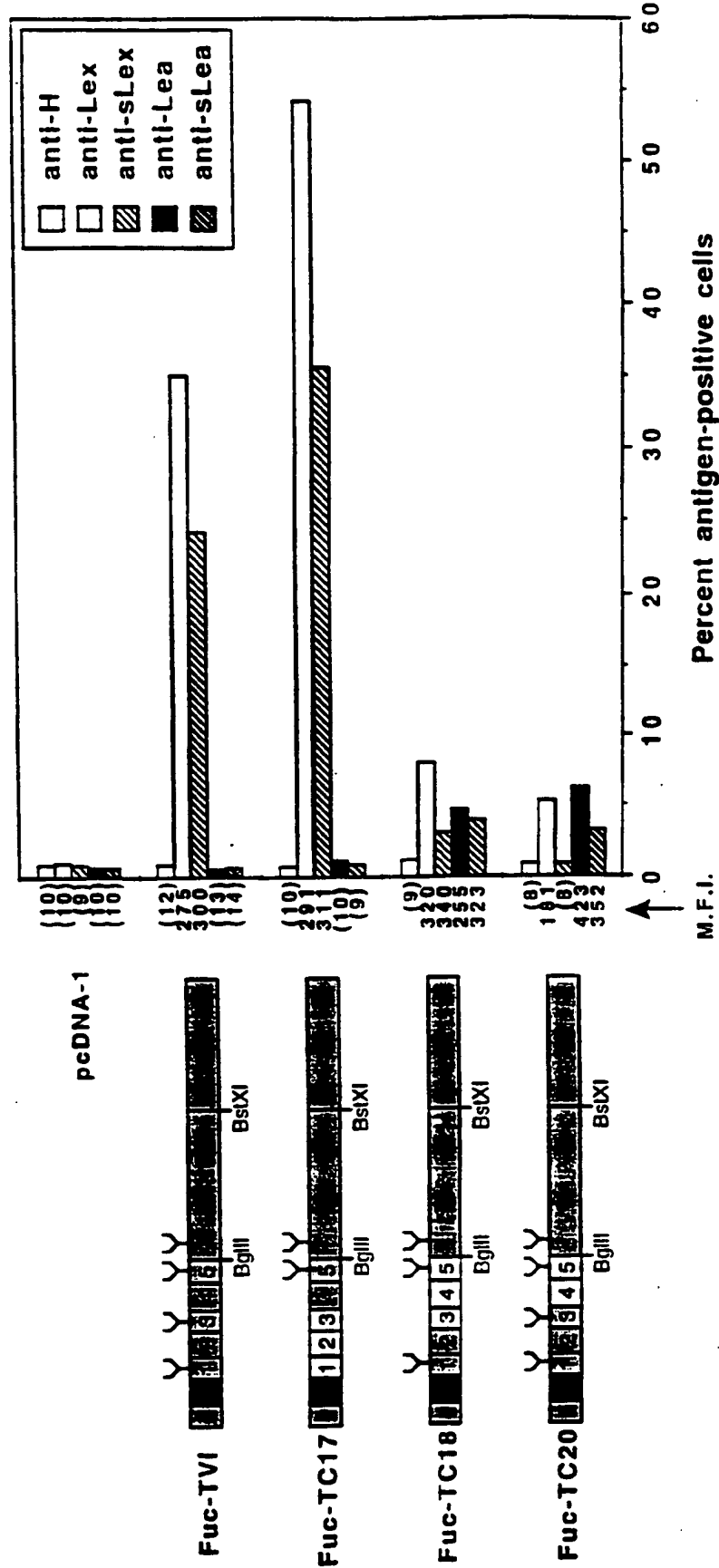
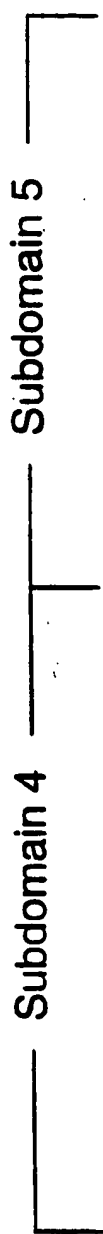


Figure 12b



Fuc-TIII 103...ADTVIVHHWDIMSNPKSRLPPSPRQGRWIWFNLEPPNCQHLEALDRYF..  
 Fuc-TV 116...-A-----Y--SAN---PT-----SM-S-S-R-----G--..

Fuc-TVI 102...--A-----REV-Y--SAQ--R---R-----SM-S-SH-WQ-K-M-G--..

"Unique" .. \*\*\*\*\*REV\*\*\*\*\*Q\*\*R\*\*R\*\*\*\*\*H\*WQ\*K\*M\*\*\*..  
 to Fuc-TVI

\* Indicates residues in Fuc-TVI that are also in either Fuc-TV, or Fuc-TIII, or both

Figure 13

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/13816

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHANDRASEKARAN et al. Expression of Blood Group Lewis b Determinant from Lewis a: Association of This Novel $\alpha(1,2)$ -L-Fucosylating Activity with the Lewis Type $\alpha(1,3/4)$ -L-Fucosyltransferase. Biochemistry. 11 April 1995, Vol. 34, No. 14, pages 4748-4756.	1-5
A	HOLMES et al. Structure-Function Analysis of Human $\alpha 1\rightarrow 3$ Fucosyltransferases. J. Biol. Chem. 07 April 1995, Vol. 270, No. 14, pages 8145-8151.	1-5
A, P	XU et al. Structure-Function Analysis of Human $\alpha 1,3$ -Fucosyltransferase. J. Biol. Chem. 12 April 1996, Vol. 271, No. 15, pages 8818-8823.	1-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

26 NOVEMBER 1996

Date of mailing of the international search report

04 DEC 1996

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Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/13816

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12N 9/10, 15/00, 5/00, 1/20; C12P 21/06; C07H 21/04

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/69.1, 193, 240.1, 252.3, 320.1; 536/23.2, 23.4

## B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

435/69.1, 193, 240.1, 252.3, 320.1; 536/23.2, 23.4

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS and STN Bioscience Index (45 Databases Searched): fucosyl transferase/, fucosyltransferase/, catalytic site/, active site/, active domain/, catalytic domain/, domain/, chimera/, fusion protein/; Seq. ID Nos.: 1, 2, 10, 11, 13 and 14: genbank, embl, ests, n-geneseq, n-geneseq, PIR, swinsprot

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